Dynamique, réactivité et écotoxicité des nanoparticules d'oxydes métalliques dans les sols : impact sur les fonctions et la diversité des communautés microbiennes

Marie Simonin

Thèse de l'Université de Lyon

Délivrée par l'Université Claude Bernard Lyon 1

Ecole Doctorale : Evolution, Ecosystèmes, Microbiologie et Modélisation

Pour l'obtention du

Diplôme de DOCTORAT en Biologie

Spécialité «Ecologie des communautés, fonctionnement des écosystèmes, écotoxicologie »

(Arrêté du 7 Août 2006)

Présentée par

Marie SIMONIN

Dynamique, réactivité et écotoxicité des nanoparticules d'oxydes métalliques dans les sols : impact sur les fonctions et la diversité des communautés microbiennes

---

Thèse dirigée par Agnès RICHAUME-JOLION et Jean MARTINS

Soutenue le 12 octobre 2015 devant le jury composé de **:**

Mme Pascale BAUDA (Professeure – Université de Lorraine)	*Rapporteur*
Mr Bruno COMBOURIEU (Professeur – Université Claude Bernard Lyon 1)	*Président du jury*
Mr Emmanuel DOELSCH (Directeur de recherche – CIRAD, Aix en Provence)	*Examinateur*
Mme Joana FALCAO-SALLES (Professeure - Université de Groningen, Pays-Bas)	*Rapporteur*
Mr Jean MARTINS (Directeur de recherche – CNRS, Grenoble)	*Co-directeur de thèse*
Mme Agnès RICHAUME-JOLION (Professeure – Université Claude Bernard Lyon 1)	*Directrice de thèse*
Mr Erik SMOLDERS (Professeur - Université de Louvain, Belgique)	*Examinateur*

Thèse mise en place et soutenue par la Région Rhône-Alpes, préparée au sein du **Laboratoire d'Ecologie Microbienne** et du **Laboratoire des Transferts en Hydrologie et Environnement**

Liste des composantes
Université Claude Bernard Lyon 1

Président de l'Université	**M. François-Noël GILLY**
Vice-président du Conseil d'Administration	M. le Professeur Hamda BEN HADID
Vice-président du Conseil des Etudes et de la Vie Universitaire	M. le Professeur Philippe LALLE
Vice-président du Conseil Scientifique	M. le Professeur Germain GILLET
Directeur Général des Services	M. Alain HELLEU

COMPOSANTES SANTE

Faculté de Médecine Lyon Est – Claude Bernard	Directeur : M. le Professeur J. ETIENNE
Faculté de Médecine et de Maïeutique Lyon Sud – Charles Mérieux	Directeur : Mme la Professeure C. BURILLON
Faculté d'Odontologie	Directeur : M. le Professeur D. BOURGEOIS
Institut des Sciences Pharmaceutiques et Biologiques	Directeur : Mme la Professeure C. VINCIGUERRA
Institut des Sciences et Techniques de la Réadaptation	Directeur : M. le Professeur Y. MATILLON
Département de formation et Centre de Recherche en Biologie Humaine	Directeur : Mme. la Professeure A-M. SCHOTT

COMPOSANTES ET DEPARTEMENTS DE SCIENCES ET TECHNOLOGIE

Faculté des Sciences et Technologies	Directeur : M. F. DE MARCHI
Département Biologie	Directeur : M. le Professeur F. FLEURY
Département Chimie Biochimie	Directeur : Mme Caroline FELIX
Département GEP	Directeur : M. Hassan HAMMOURI
Département Informatique	Directeur : M. le Professeur S. AKKOUCHE
Département Mathématiques	Directeur : M. le Professeur Georges TOMANOV
Département Mécanique	Directeur : M. le Professeur H. BEN HADID
Département Physique	Directeur : M. Jean-Claude PLENET
UFR Sciences et Techniques des Activités Physiques et Sportives	Directeur : M. Y.VANPOULLE
Observatoire des Sciences de l'Univers de Lyon	Directeur : M. B. GUIDERDONI
Polytech Lyon	Directeur : M. P. FOURNIER
Ecole Supérieure de Chimie Physique Electronique	Directeur : M. G. PIGNAULT
Institut Universitaire de Technologie de Lyon 1	Directeur : M. le Professeur C. VITON
Ecole Supérieure du Professorat et de l'Education	Directeur : M. le Professeur A. MOUGNIOTTE
Institut de Science Financière et d'Assurances	Directeur : M. N. LEBOISNE

Table des matières

Table des illustrations

Table des illustrations

Remerciements

Je m'excuse par avance pour la longueur de cette section remerciements. J'ai souhaité cette partie la plus exhaustive possible, car je n'ai pas toujours pris le temps de remercier comme il se fallait toutes les personnes qui m'ont permis d'arriver jusqu'ici et de mener à bien cette thèse.

Je remercie l'ensemble des membres du jury pour avoir accepté d'évaluer mes travaux, en particulier mes rapporteurs Pascale Bauda et Joana Falcão Salles.

Je commencerai par remercier Agnès et Jean qui m'ont fait confiance pour réaliser ces travaux. J'ai adoré faire cette thèse avec vous et si je pouvais je recommencerais bien !

Agnès, j'ai du mal à trouver les mots pour t'exprimer ma reconnaissance pour ces 3 années de thèse sous ton encadrement. Je ne te remercierais jamais assez pour m'avoir accordé ta confiance aussi rapidement et de m'avoir fait découvrir tout l'univers de la recherche, ainsi que le métier d'enseignant-chercheur. J'ai beaucoup appris sur l'encadrement, la rédaction et la création de projet de recherche grâce à toi et je sais à présent que c'est ce métier que je veux faire plus tard. Désolé pour tout le travail que je t'ai envoyé les soirs, week-ends et vacances, et merci pour ta réactivité, disponibilité et patience pour gérer mes ardeurs scientifiques. En exclusivité intergalactique rien que pour toi, un GRAND MERCI !

Jean, je te remercie d'avoir toujours su trouver un moment pour moi quand j'étais de passage à Grenoble. J'ai fait de mon mieux pour réussir à faire une vraie thèse pluridisciplinaire, même si tu sais mieux que quiconque toutes les difficultés que cela représente. Je partais de zéro pour l'étude du transport et de la caractérisation des polluants dans les sols, merci de m'avoir fait découvrir le monde de la biogéochimie et pour ta pédagogie et ta patience.

Je tiens à remercier les membres de mon comité de pilotage : Bruno Combourieu, Robert Duran, Xavier Le Roux, Jérome Rose, Géraldine Sarret et Laurent Simon, qui ont suivi le déroulement de ces 3 ans de thèse. Je les remercie pour leurs conseils qui ont permis de réaliser des choix stratégiques pertinents durant la thèse.

Remerciements

Je voudrais adresser un remerciement également au personnel administratif des 2 laboratoires que j'ai beaucoup sollicité pendant ces 3 années, en particulier au LEM : Dominique, Stéphane, Christine, Betty, et au LTHE Claire, François, Odette, Valérie, Nathalie et Carméline.

LTHE – Grenoble

Je tiens à remercier les membres du LTHE, en particulier de l'équipe TRANSPORE, nouvellement nommée HyDRIMZ, qui m'ont accueilli régulièrement pendant 3 ans et surtout pendant 6 mois lors de ma 2eme année.

Un grand merci à Aline pour ses conseils pour les colonnes et la chromato. Merci à Gaëlle et Marie-Christine pour leur patience et leur aide pour gérer notre micro-onde de malheur. Gaëlle, merci encore pour m'avoir logée et d'avoir toujours su trouver le temps pour les extractions et les articles. Alix et Aude, nos 2 supers thésardes, merci pour votre aide lors de ma fin de thèse, respectivement pour Stanmod et les mesures de potentiels oxydants ! Merci également à Erwann et Sophie pour leur aide de tous les jours et leurs bonnes humeurs. Je n'oublie pas Fred, Dénisse, Lorenzo, Céline, Jerusa, Alex, Ana, Jean-Paul… pour la bonne ambiance et l'entraide au labo.

Labo Ecologie Microbienne - Lyon

J'ai passé 3 années mémorables au LEM au sein de l'équipe 5 Diversité fonctionnelle microbien et cycle de l'azote. J'ai eu la chance de travailler dans de très bonnes conditions (mise à part l'état des locaux, vivement le plan campus !) et aux côtés de supers scientifiques. J'ai bien peur de ne pas pouvoir retrouver une telle ambiance de travail dans le futur…

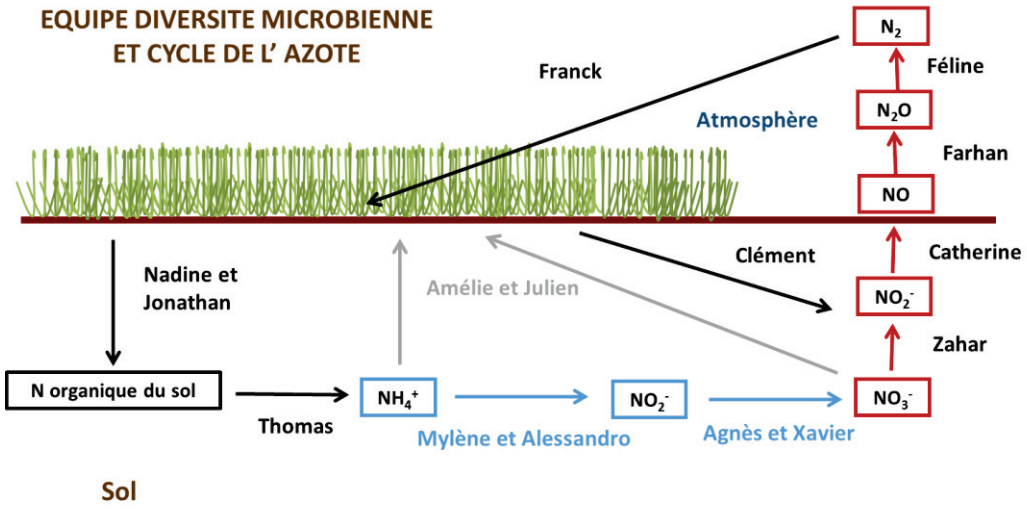
Merci à mes Co-bureau (418 I yeah !): On a des stores cassés et pas de serrure mais on travaille dans la bonne humeur ! Merci à MAP, Corinne, Brigitte, Catherine, Nadine, Agnès et Alessandro pour ces heures partagées ensemble. Désolé si je vous ai parfois effrayé avec mes bonjours et bonnes années un peu trop enthousiaste !

Remerciements

Equipe 5

Je tiens à remercier les membres de l'équipe 5 pour leur accueil et leur aide et également pour nos supers thématiques de recherche sur le cycle de l'N et plus généralement en écologie. J'ai beaucoup appris à vos côtés. Un petit bisou spécial à Amélie qui partage avec moi la passion des plantes et des sorties à Botanic, mon objectif après la thèse c'est d'avoir un aussi beau papyrus que toi !

Vous trouverez ci-dessous une représentation schématique de l'organisation de l'équipe qui assure la délivrance de services écosystémiques durables et d'un tout petit peu de gaz à effet de serre ! Les emplacements des personnes dans le cycle sont parfois aléatoires et parfois pleinement justifiés.



Je tiens à adresser un grand merci à mes supers stagiaires, sans vous cette thèse n'aurait pas été possible. Il s'agissait d'une première expérience d'encadrement pour moi et j'ai beaucoup apprécié travailler avec vous. Je vous souhaite le meilleur à tous pour le futur ! Par ordre chronologique d'apparition : Julien, Morgane, Pauline, Florian, Mathilde, Mia (x2), Armelle et Sofia.

iBio

Merci à la plateforme iBio pour leur aide dans l'analyse du séquençage. En particulier à Audrey Dubost pour sa patience, ses conseils et sa bonne humeur. Il aura fallu 1 an et demi mais on y est arrivé ! Euh au fait j'ai un congrès en mai, tu penses que ça va le faire ??

Remerciements

Les étudiants

Je pense que j'ai eu la chance de faire partie d'une des générations dorées (Jeanne pourra le confirmer ou non :p) des étudiants du laboratoire d'écologie microbienne ! Sur le plan scientifique, il existe une grande entraide entre les étudiants et une volonté d'organiser le laboratoire pour améliorer la qualité des expérimentations, l'accueil des étudiants et la sécurité. En dehors du labo, les initaitives n'ont également pas manqué pour organiser des sessions de sport (fouting, volley - LEMuriens, foot – FC LEM) et des barbec ou aller au King Kong ! J'espère de tout cœur que cette dynamique perdurera sur le long terme grâce à nos représentants étudiants et la bonne volonté des thésards et stagiaires.

Merci à tous ! Et par ordre alphabétique en particulier à :

Antoine, philosophe, guitariste de talent, Schwartz Tiger, le chocothésard que tout le monde rêve d'avoir ! On te doit beaucoup pour la super ambiance au labo.

Clément, merci pour les supers discussions scientifiques sur le fractalité de l'univers et aussi pour les discussions sur DBZ, star wars… Bonne chance pour la suite, le Bardon Denitrification Index règnera sur le monde !

David, oui je t'ai mis dans la section étudiant, c'est toujours flatteur à ton âge !

Florian, la seule chose au monde qui fait que Blues, Ninjutsu, Moyen-Age, Chevalier et Laspallès, et champignons ont un point commun ! C'était trop cool la musique, tu es le bienvenu pour boire un Picon à la maison quand tu veux.

Guillaume, j'ai voté pour ta canonisation en 2016 ! Merci pour tes précieux conseils en heatmap, mothur et compagnie. Bonne chance pour la suite au pôle nord, je pense que Albo te laissera un peu tranquille à cette température.

Jojo, le champion de la blague la plus Normale d'après mon test de Shapiro ! Je souhaite de tout cœur que le grand Cas-Anova ait l'occasion de plotter une Pearson prochainement ! Merci beaucoup pour toutes nos discussions scientifiques ou non, entraide en bio mol et stats, initiation au billard et au foot. RDV dans quelques années pour notre projet SWAG qui révolutionnera les PGPR. Tu iras loin Jojo, j'ai confiance dans tes placements dans la Pseudo Banque (Gnéééé)!

Remerciements

Julien, mon 1er stagiaire (tu seras toujours mon préféré) ! Rourou le siffleur fou, pense à réduire le nombre de pause-café en 3eme année !

Lise, Ma coloc adorée. Ta présence aura été très bénéfique durant cette dernière année de thèse, merci. Courage pour mobi**Lise**r les troupes de notre super asso, réa**Lise**z de belles découvertes scientifiques dans la grotte de Lascaux et tota**Lise**r un nombre de but sans précédent au sein du FC LEM !

Seb, merci d'avoir partagé avec moi les galères de qPCR, ça fait du bien de savoir qu'on n'est pas seul à lutter ! Courage pour *phlD*, au pire comme reconversion pense à la reprographie -> la plastifieuse ça a de l'avenir. Un seul conseil pour le bon déroulement de ta fin de thèse : B***** des meufs avec l'habitat Renoud de préférence!

Yo BM, le Titi Henry du FC LEM ! Merci pour ton investissement dans la vie du labo, la relève est bien là ! J'espère que tu ne m'oublieras pas pour me raconter tous les potins du labo.

Je n'oublie pas les anciens Max, Stéphanie, Benoit, Marie, Benjamin Y, Laetitia, Adrien, Florent, Cyrus, Malek…

Bonne chance à tous les petits nouveaux Quentin, Elodie, Zazou, Thibault, Guillaume S, Aurélie, Rosa, Tania, Marine, Florian, Jordan…, profitez bien de ces années au labo. Je reviendrais régulièrement pour vérifier que l'ambiance reste au beau fixe !

Je salue mes collègues du foot, le seul endroit où on se mélange avec les gens du CESN et de l'équipe 6 (Troll :p) : Max, Romain, Guillaume, Serge, Slim, PE, PA, Amine, les autres sont déjà cités avant.

Asso Doc E2M2

J'ai eu le plaisir d'être la secrétaire de l'association des doctorants E2M2 aux côtés de gens supers n'appartenant pas forcément au LEM (eh oui c'est possible) comme Christophe, Kevin, Ben, Mag, Manon, Melissa, Jean, Nicolas, Wilhelm… Notre asso a de l'avenir et j'ai l'espoir que les doctorales et les gens du jardin, ainsi que nos futures tartiflettes scientifiques deviennent les évènements les plus prisés de tout Lyon !

J'en profite pour remercier Gudrun Bornette et Hubert Charles pour leur soutien ainsi que Fabrice Cordey et Sylvie Reverchon nos supers nouveaux directeurs !

L'avant-thèse

Je tiens à remercier toutes les personnes qui m'ont permis d'arriver ici aujourd'hui, en particulier mes différents encadrants de stage de licence et de master qui m'ont fait découvrir la recherche. Un grand merci à Audrey Niboyet, pour son encadrement pendant mes 2 stages de Master. J'ai acquis de solides bases grâce à toi qui m'ont beaucoup aidé durant la thèse et ça a toujours été un vrai plaisir de travailler à tes côtés ! Je remercie également Jean-Marie Ballouard et Xavier Bonnet qui m'ont encadré lors de mes stages de licence qui portaient sur l'écologie des serpents. J'ai fait pas mal de chemin depuis les capture-marquage-recapture à Chizé ou en Nouvelle-Calédonie…

Je remercie Elisa qui m'a permis de trouver mon stage de M1 avec Audrey, ce qui a été le catalyseur de toutes les bonnes choses qui me sont arrivées ensuite !

La famille et les amis

Merci aux amis lyonnais, parisiens, normands et vosgiens sans qui les week-ends et les vacances ne seraient pas les mêmes : Pippo, Audrey, Coco, Dédé, Pedro, Jo, So, Chinois, Jess, Nico, Julie, Marion, Laurent, Minouche, Papi, Jordan, Camille, Clémence…

Je remercie la famille Geissler si je suis là aujourd'hui c'est aussi grâce à vous !

Laurent (a.k a Lol), je tiens à te remercier pour toutes ses années d'amitiés et pour m'avoir redonné confiance en moi en 3ème, ça peut paraître ridicule dit comme ça mais si tu n'avais pas été là je pense que je n'aurai pas fait le même parcours !

Charlotte, 21 ans d'amitié (on est des vieux machins…), difficile de mettre des mots pour exprimer tout ce que je te dois. Ma vie serait bien moins heureuse sans les heures passées ensemble dans la petite maison, le CD tournesol, le coude dans le plat, Pitbull, les bouteilles

de champagne de trop, les horizontales ou tes tiramisus. Un bisou à tous les Rousseau/Borys au passage !

Je remercie toute ma famille pour leurs encouragements et la bonne humeur qui règne, les noëls, les vacances à Oléron, la cousinade à Lyon…

Un immense merci à mes parents pour leur soutien pendant toutes ses années. Merci de m'avoir toujours fait confiance pendant toutes mes études, je vous dois tout. Merci maman, pour l'intérêt que tu as toujours porté à ce que je faisais pendant mes études, pour tous les rapports et articles que tu as relu de manière très assidue pour m'aider.

Par-dessus tout, merci à Théo. Il ne fallait pas moins que le fils de Vegeta pour m'accompagner dans cette aventure ! Merci pour tous les bons moments ensemble, ta passion partagée de la gastronomie (Lyon va nous manquer), ta patience et ton soutien sans faille.

Je tiens également à remercier sincèrement les Foo Fighters qui m'ont accompagné pendant toute la rédaction de cette thèse. Dave Grohl si tu lis cette thèse, Merci !

Résumé

Les nanoparticules métalliques manufacturées (NPs) sont des polluants émergents dont la concentration augmente dans les sols en raison de leur utilisation croissante dans de nombreux produits commerciaux de la vie courante (cosmétiques, aliments, peintures…). Des études *in vitro* ont montré la toxicité des NPs pour les microorganismes, mais il existe encore peu de données sur l'écotoxicité et le devenir de ces contaminants dans les sols.

L'objectif de cette thèse est donc d'évaluer l'influence des paramètres abiotiques du sol sur (i) les caractéristiques physico-chimiques et le transfert des NPs, et (ii) sur la toxicité des NPs pour les communautés microbiennes du sol, en particulier pour des groupes fonctionnels microbiens impliqués dans le cycle du carbone et de l'azote.

Nous avons mis en évidence que les propriétés du sol influençaient l'agrégation et la charge de surface des NPs de dioxyde de titane (TiO_2) et d'oxyde de cuivre (CuO). Dans les six sols agricoles étudiés, nous avons observé un transport faible (CuO) à très faible (TiO_2) des NPs testées lors d'une expérimentation en colonnes de sols. Une étude en microcosmes de sol a permis de mettre en évidence une absence de toxicité des NPs de TiO_2 sur les communautés microbiennes, sauf dans un sol limono-argileux à forte teneur en matière organique. Dans ce sol, des effets négatifs ont été observés après 90 jours d'exposition sur les activités microbiennes (respiration, nitrification et dénitrification), sur l'abondance des microorganismes nitrifiants et la diversité des bactéries et des archées. De plus, nous avons montré que les effets négatifs observés sur la nitrification, même pour des concentrations extrêmement faibles de TiO_2 (0.05 mg kg^{-1}), étaient liés principalement à une forte sensibilité à ce polluant des archées oxydatrices de l'ammonium (AOA) impliquées dans ce processus. Des études complémentaires en colonnes de sol, ont permis de mettre en évidence des effets délétères des NPs plus importants sur la nitrification lors d'une contamination chronique au TiO_2 que lors d'une contamination aigüe.

Nous avons ainsi montré que la toxicité des NPs métalliques est réelle dans les sols et qu'elle est influencée par leurs propriétés physico-chimiques. Des processus microbiens clés comme la nitrification peuvent être fortement altérés par la présence de NPs. La rétention importante des NPs dans les sols suggère que ces polluants peuvent persister durablement dans les sols. Dans ce contexte, l'exposition chronique des sols sur le long terme doit être prise en compte dans des expérimentations visant à évaluer les risques environnementaux liés à la présence de ces polluants émergents.

Abréviations

AOA : Archées oxydatrices de l'ammonium

AOB : Bactéries oxydatrices de l'ammonium

Br : Brome (Bromide)

CCC : Concentration Critique de Coagulation

CEC : Capacité d'Echange Cationique

CNT : Carbon Nanotube

CuO : Oxyde de cuivre (copper oxide)

DCFH : 2',7'-dichlorofluorescein

DEA : Denitrification Enzyme Activity

DOC : Dissolved Organic Carbon

DLS : Dynamic Light Scattering

ICP-OES : Inductively Coupled Plasma - Optical Emission Spectrophotometer

MO : Matière Organique

MWCNT : Multi-walled carbon nanotube

NEA : Nitrification Enzyme Activity

NMDS : Non-Metric multi-Dimentional Scaling

NOB : Bactéries oxydatrices du nitrite

NP : Nanoparticule

OM : Organic Matter

OP : Oxidative potential

OTU : Operational Taxonomic Unit

PV : Pore Volume

qPCR : quantitative Polymerase Chain Reaction

ROS : Espèce réactive de l'oxygène (Reactive Oxygen Species)

SIR : Substrate-Induced Respiration

SWCNT : Single-Walled Carbon Nanotube

TiO_2 : Dioxyde de titane (titanium dioxide)

WHC: Water Holding Capacity

Introduction générale

Les écosystèmes terrestres sont soumis en permanence à des perturbations d'origines anthropiques (e.g. pratiques agricoles, pollutions, compaction, salinisation). Les sols constituent un des principaux compartiments d'accumulation des polluants provenant d'origine diverses (irrigation, déversement accidentel, épandage de boues de station d'épuration, produits phytosanitaires, déposition atmosphérique…). De nouveaux types de contaminants qualifiés d'émergents, sont identifiés régulièrement dans les sols suite à la commercialisation de nouveaux produits pour lesquels il n'existe pas encore de réglementation et dont les effets environnementaux ne sont pas connus. Suite à l'avènement des nanotechnologies au début des années 2000, des inquiétudes ont émergé concernant le devenir et la toxicité des nanomatériaux dans l'environnement. En effet, les nanomatériaux présentent des propriétés exceptionnelles attisant l'intérêt des industriels (e.g. photo-catalytique, optique, semi-conducteur, antimicrobien). Cependant, ces propriétés peuvent, dans certains cas, leur conférer un potentiel toxique démontré en conditions *in vitro*. A ce jour, l'impact de ces polluants émergents a été étudié majoritairement dans les écosystèmes aquatiques, alors que les sols représentent vraisemblablement l'écosystème le plus exposé aux nanomatériaux.

En écotoxicologie, les sols reçoivent peu d'attention alors qu'ils rendent un très grand nombre de services écosystémiques clés parmi lesquels la production alimentaire, la qualité de l'eau et la régulation du climat qui font partie de grands enjeux socio-économiques actuels. Le sol est un système hétérogène complexe composé de fractions minérales, organiques, aqueuses et gazeuses. La complexité de l'étude des sols est également liée à la grande diversité de sols, en termes de texture, pH, ou encore de teneur et nature de matière organique qui sont autant de paramètres connus pour influencer le devenir et la toxicité des polluants. Dans une approche d'évaluation des risques, il est donc nécessaire de considérer l'influence des propriétés du sol sur les caractéristiques physico-chimiques du polluant, sur sa mobilité pour déterminer les risques de son transport et de sa dispersion en dehors du site de pollution initial, ainsi que sur sa biodisponibilité et sa toxicité.

Les sols hébergent de nombreux et divers organismes (macrofaune, mésofaune, microorganismes). Parmi eux les communautés microbiennes représentent une biodiversité considérable jouant un rôle fondamental dans les cycles biogéochimiques et la dégradation des polluants. Pour évaluer la qualité d'un sol et sa réponse à une perturbation, l'activité,

l'abondance et la diversité des communautés microbiennes sont reconnues comme étant des indicateurs pertinents. En effet, les communautés microbiennes sont sensibles à de nombreuses perturbations qui peuvent altérer leur activité et par conséquent avoir des conséquences notables sur le fonctionnement de leur écosystème. De plus, cette approche permet d'étudier l'impact d'un contaminant à l'échelle d'une communauté d'organismes du sol et non pas uniquement sur une population bioindicatrice comme cela est fait plus classiquement en écotoxicologie.

De ce fait, afin d'évaluer les risques associés à des contaminants émergents tels que les nanomatériaux, une approche couplée abordant l'écodynamique du polluant dans le sol et son impact sur des indicateurs écologiques pertinents est nécessaire. C'est dans ce contexte qu'a été initiée cette thèse en lien avec un projet de recherche intitulé : « *Les nanoparticules métalliques dans les écosystèmes terrestres : écodynamique et impact sur les communautés bactériennes dans les sols* ». Ce projet a fait intervenir 2 laboratoires : Ecologie Microbienne (Lyon 1 - CNRS – INRA) et LTHE (Grenoble 1 – CNRS – G-INP - IRD) et a été financé par le programme national EC2CO (Ecosphère Continentale et Cotière) Microbien.

L'objectif principal de cette thèse était d'étudier l'influence des propriétés du sol sur la mobilité des nanoparticules de dioxyde de titane (TiO_2) et d'oxyde de cuivre (CuO) et leur toxicité sur des communautés microbiennes choisies en raison de leur rôle central dans la fertilité des sols. Les effets des nanoparticules sur des communautés microbiennes impliquées dans le cycle de l'azote (nitrifiants et dénitrifiants) ont donc été considérés afin d'évaluer la réponse de groupes fonctionnels microbiens présentant différents niveaux de redondance fonctionnelle. De plus, afin de déterminer l'influence du mode d'exposition sur le devenir et la toxicité des nanoparticules, des contaminations aigües et chroniques ont été étudiées.

Ce travail est organisé en 4 chapitres :

- Le premier chapitre est une synthèse bibliographique concernant le devenir des nanomatériaux dans l'environnement et leur impact sur les communautés microbiennes qui fait l'objet d'un article publié. De cet état de l'art ont découlé les questionnements de la thèse et les choix méthodologiques pour mener à bien ce travail.

- Dans le second chapitre sont présentés les résultats concernant l'influence des propriétés du sol sur le transport du TiO_2 et CuO, ainsi que sur la toxicité du TiO_2 sur les communautés microbiennes sous forme de deux articles.
- Dans le troisième chapitre sont présentés sous forme de trois articles, les résultats de l'impact du TiO_2 sur les communautés nitrifiantes et dénitrifiantes dans un sol limono-argileux lors de contaminations aigues ou chroniques.
- Le quatrième chapitre est une conclusion générale sur l'ensemble des résultats obtenus. A la lumière de ces conclusions, les perspectives de recherche qui nous semblent les plus pertinentes sont proposées pour l'étude du devenir et de l'impact des nanomatériaux dans les sols.

La présentation des résultats de la thèse s'organise selon le schéma général présenté ci-dessous (Figure 1).

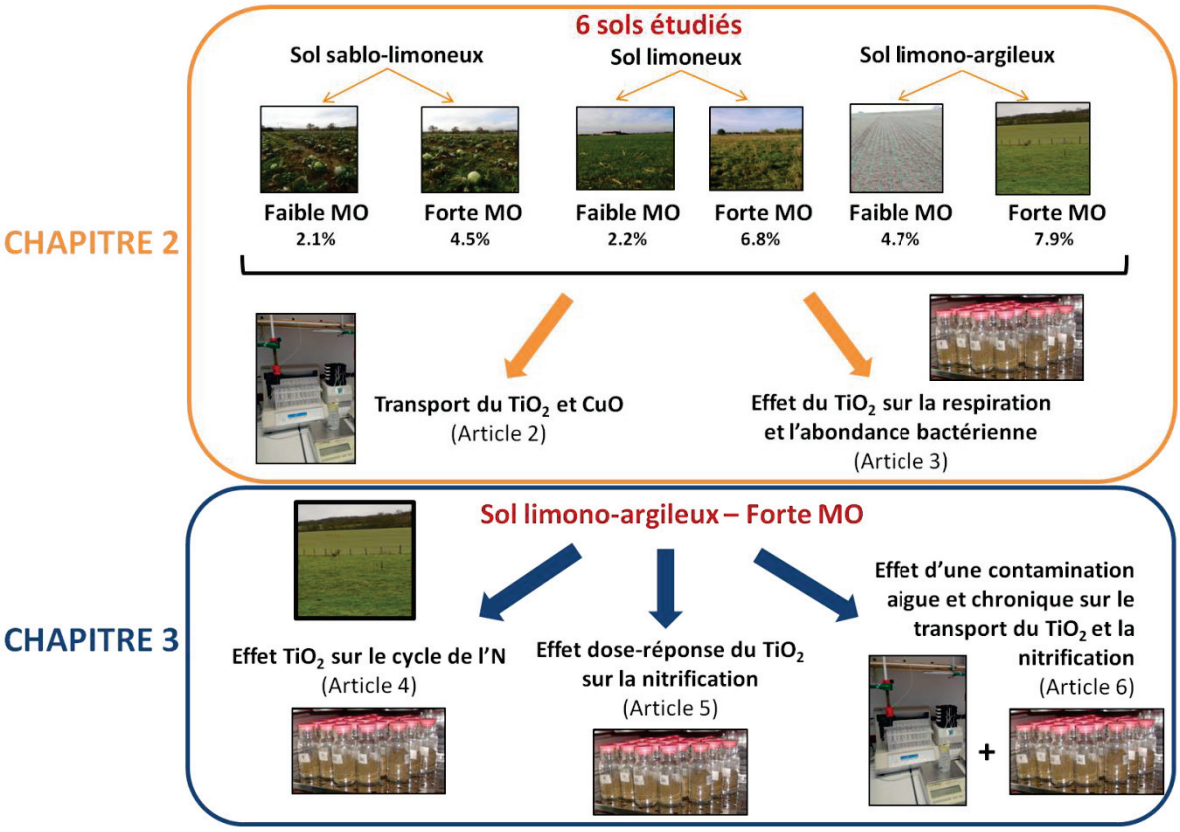


Figure 1: Schéma général d'organisation des résultats de la thèse.

Chapitre 1: Synthèse bibliographique, questions et choix méthodologiques

1. Les nanomatériaux, contaminants émergents des écosystèmes terrestres

a. Définitions

Un nanomatériau est défini comme *"un matériau naturel, formé accidentellement ou manufacturé contenant des particules libres, sous forme d'agrégat ou sous forme d'agglomérat, dont au moins 50 % des particules, dans la répartition numérique par taille, présentent une ou plusieurs dimensions externes se situant entre 1 nm et 100 nm"* (Figure 2, Commission européenne, 2011).

Parmi ces nanomatériaux, on distingue différentes familles de nano-objets manufacturés (INRS, 2012) :

- **les nanoparticules (NPs)** qui désignent des nano-objets dont les trois dimensions externes se situent à l'échelle nanométrique : nanoparticules de latex, d'oxyde de zinc, de fer et de cérium, d'alumine, de dioxyde de titane, de carbonate de calcium, etc ;

- l**es nanofibres, nanotubes, nanofilaments ou nanobâtonnets** qui se rapportent à des nano-objets dont deux dimensions externes sont à l'échelle nanométrique et la troisième dimension significativement supérieure. Ces termes désignent des nano-objets longilignes de section comprise entre 1 et quelques dizaines de nm et de longueur comprise entre 500 et 10 000 nm comme, par exemple, les nanotubes de carbone, les nanofibres de polyester, les nanotubes de bore, etc…;

- **les nano-feuillets, nano-plats ou nano-plaquettes** qui définissent des nano-objets dont une dimension externe se situe à l'échelle nanométrique, les deux autres étant significativement supérieures. C'est le cas des nano-feuillets d'argile, des nano-plaquettes de séléniure de cadmium.

Les nanomatériaux peuvent donc se présenter sous un grand nombre de formes différentes, mais ils sont également caractérisés par une grande variété de propriétés chimiques. On peut distinguer les nanomatériaux en fonction de la nature organique ou inorganique de l'élément chimique les constituant. Les nanomatériaux organiques sont majoritairement commercialisés sous forme de nanotubes de carbone ou de fullerènes. Les nanomatériaux

inorganiques sont divisés en 3 classes : les nanomatériaux métalliques (argent, or…), les oxydes métalliques (titane, fer, cérium, cuivre…), et les quantum-dots (séléniure de cadmium) (Ju-Nam and Lead, 2008).

Les nanomatériaux ne peuvent donc pas être considérés comme un seul groupe homogène de contaminants, et leur diversité doit être prise en compte pour l'évaluation des risques liés à ces composés qui sont incorporés dans de nombreux produits industriels et commerciaux depuis une vingtaine d'années.

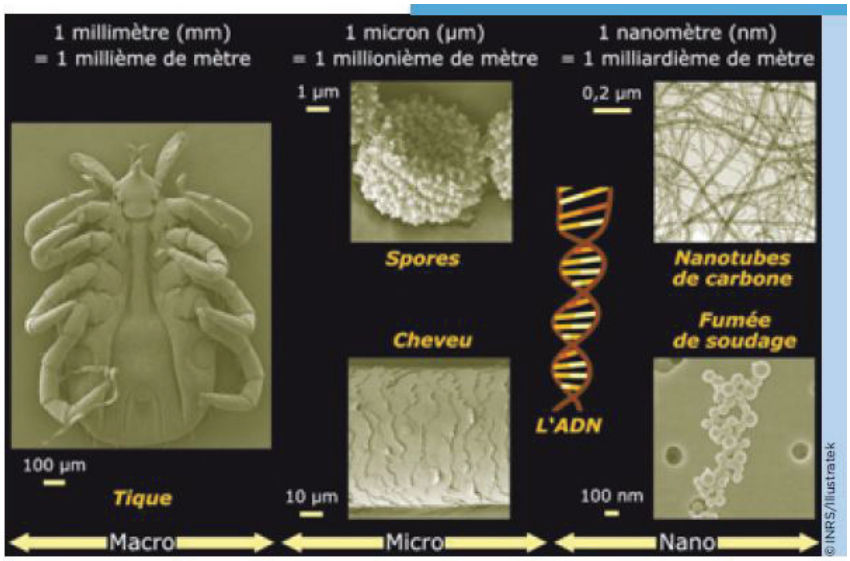


Figure 2: L'échelle des dimensions : du visible à l'invisible (INRS, 2015)

b. Utilisation des nanomatériaux dans des produits de consommation

Les nanomatériaux sont caractérisés par un pourcentage élevé d'atomes en surface ce qui leur confèrent des propriétés nouvelles et une forte réactivité comparées à celles des matériaux de même composition chimique mais de plus grande taille (Auffan *et al.*, 2009). Des propriétés électriques, optiques ou catalytiques par exemple, sont ainsi modifiées à l'échelle nanométrique. Ceci accorde aux nanomatériaux des propriétés physico-chimiques d'intérêt dans le milieu industriel et explique que l'ère des nanotechnologies est souvent décrite comme ouvrant une nouvelle révolution industrielle. Le marché des nanomatériaux est en effet estimé à 1 milliard de dollars en 2015 (Nel *et al.*, 2006).

Les nanomatériaux manufacturés sont déjà utilisés dans des domaines très variés tels que l'agriculture, l'automobile, les cosmétiques, la construction, l'énergie, l'électronique, le médical, les peintures, les produits alimentaires, la remédiation, ou encore les textiles (Keller *et al.*, 2013; Mitrano *et al.*, 2015) (Figure 3). Actuellement on estime que la NP la plus utilisée dans l'industrie est le dioxyde de titane (TiO_2) (Robichaud *et al.*, 2009; Sun *et al.*, 2014). Ses propriétés photo-catalytiques exceptionnelles permettent en effet son utilisation dans de très nombreux secteurs d'activités (Chen and Mao, 2007). Cependant, le constat de cette utilisation croissante des nanomatériaux dans des produits de la vie courante implique la connaissance de leur cycle de vie mais aussi celle de leur devenir dans l'environnement.

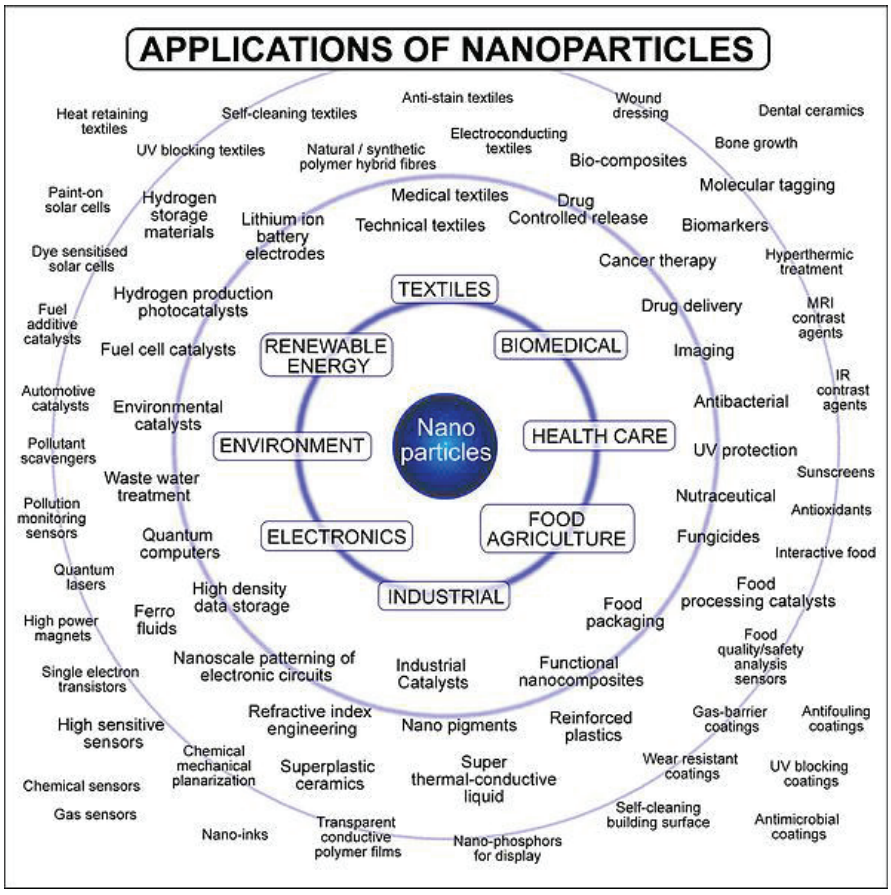


Figure 3: Exemples d'utilisation des nanoparticules dans des domaines divers (Tsuzuki, 2009).

c. Cycle de vie des nanomatériaux et rejet dans l'environnement

L'utilisation croissante de nanomatériaux soulève des interrogations quant à leur rejet dans l'environnement au cours des différentes étapes de leur cycle de vie, depuis la production jusqu'au retraitement (Figure 4).

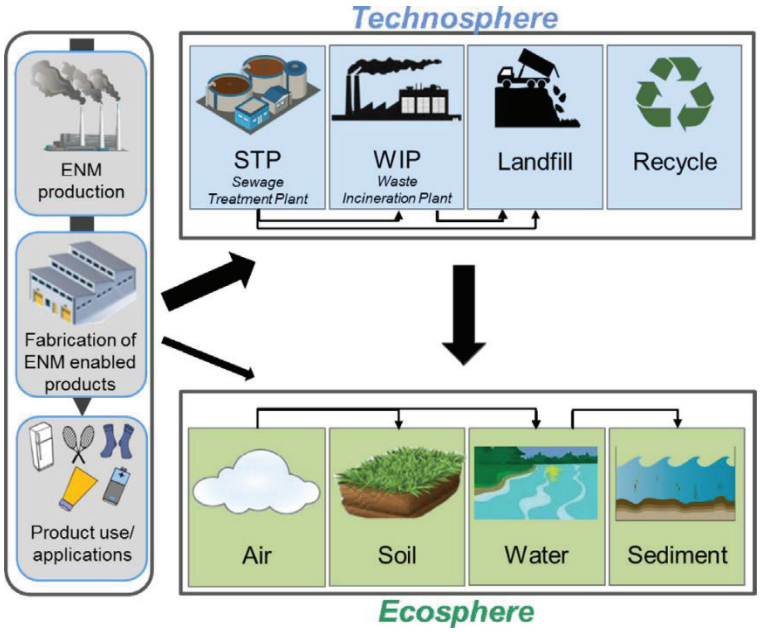


Figure 4: Cycle de vie des nanomatériaux manufacturés dans l'environnement, ENM : Engineered Nanomaterial (Sun *et al.*, 2014)

Som *et al.*, (2010) ont souligné l'importance de considérer le cycle de vie des nanomatériaux pour chaque produit afin d'obtenir une évaluation fiable des risques environnementaux associés. En effet, la forme sous laquelle seront rejetés les nanomatériaux dépendra de l'étape du cycle de vie du produit concerné (Nowack *et al.*, 2012) : (i) purs lors de la production, (ii) associés à d'autres composés lors de leur utilisation, (iii) associés à des composés environnementaux lors de leur entrée dans les écosystèmes (Figure 5).

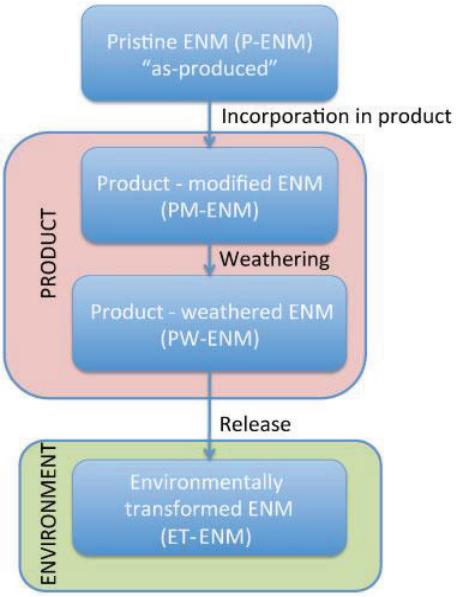


Figure 5: Transformations des nanomatériaux manufacturés au cours de leur utilisation et leur rejet dans l'environnement, ENM : Engineered Nanomaterial (Nowack *et al.,* 2012).

Dans ce contexte, de nombreuses études ont été conduites pour étudier le vieillissement et les transformations des nano-produits au cours de leur cycle de vie (Mitrano *et al.*, 2015). Plusieurs travaux ont mis en évidence que des nanomatériaux pouvaient être rejetés de manière involontaire dans l'environnement suite à leur utilisation dans des produits couramment utilisés comme les peintures, les textiles ou les cosmétiques (Labille *et al.*, 2010; Nowack *et al.*, 2012). Le relargage de TiO_2-NPs à partir des peintures appliquées sur des façades extérieures a été montré par Kaegi *et al.*, (2008) qui ont mis en évidence un transfert de concentrations importantes (3,5 10^7 TiO_2 particules/L de taille inférieure à 100 nm) vers les eaux de surfaces dans des conditions météorologiques naturelles. Il a été également estimé que 75 à 95% des nanomatériaux contenus dans les cosmétiques et crèmes solaires (e.g. TiO_2 et ZnO) étaient libérés dans l'environnement suite à une douche ou une baignade (Keller *et al.*, 2013). Ces différents exemples suggèrent que les nanomatériaux entrent involontairement dans les écosystèmes aquatiques et terrestres où ils constituent des pollutions chroniques.

Il existe également des cas où l'environnement est exposé de manière volontaire à des nano-produits, en particulier dans le cadre d'activités liées à l'agriculture ou à la remédiation des écosystèmes pollués. En effet, en agronomie de plus en plus de nanofertilisants et nanopesticides sont proposés sur le marché pour augmenter les rendements de production

végétale et lutter contre des phytopathogènes (Liu and Lal, 2015; Servin *et al.*, 2015). Les nanomatériaux présentent également une grande efficacité pour la remédiation des écosystèmes contaminés aux métaux lourds, polluants organiques et substances biologiques (virus, bactéries, antibiotiques). Ils sont ainsi utilisés pour le traitement des eaux usées, des déchets industriels ou de l'air (Hua *et al.*, 2012; Khin *et al.*, 2012).

A cause de limitations techniques, il est encore très difficile de détecter les nanomatériaux et d'en mesurer les concentrations dans des milieux environnementaux complexes (Tourinho *et al.*, 2012; Cornelis *et al.*, 2014). Cependant, comme décrit ci-dessus, il existe des preuves que les nanomatériaux sont déjà présents dans de nombreux environnements et que leurs concentrations environnementales augmentent au cours du temps.

Tableau 1: Prédiction des concentrations en TiO_2-NPs dans différents compartiments environnementaux dans les pays de l'union européenne (Sun *et al.*, 2014).

Environnement	Concentration en TiO_2 prédite
Air	0.001 µg m^{-3}
Eaux de surface	0.53 µg L^{-1}
Sédiments	1.9 mg kg^{-1} par an
Boues de station d'épuration	170 mg kg^{-1}
Sol naturel et urbain	0.13 µg kg^{-1} par an
Sol traité avec boues de station d'épuration	1.2 mg kg^{-1} par an

Sur la base de données de production annuelle de nanomatériaux et du cycle de vie des produits, des études ont permis de modéliser les concentrations environnementales des nanomatériaux les plus couramment utilisés. Plusieurs études concordent sur le fait que le nanomatériau ayant la plus forte concentration dans les différents compartiments de la technosphère et de l'écosphère est le TiO_2 (Tableau 1 et Figure 6). De plus, ces travaux montrent que les sols et les sites de stockage constituent les réceptacles environnementaux majoritaires alors qu'une fraction beaucoup plus faible est rejetée dans les écosystèmes aquatiques et dans l'air (Gottschalk *et al.*, 2009; Keller *et al.*, 2013; Sun *et al.*, 2014).

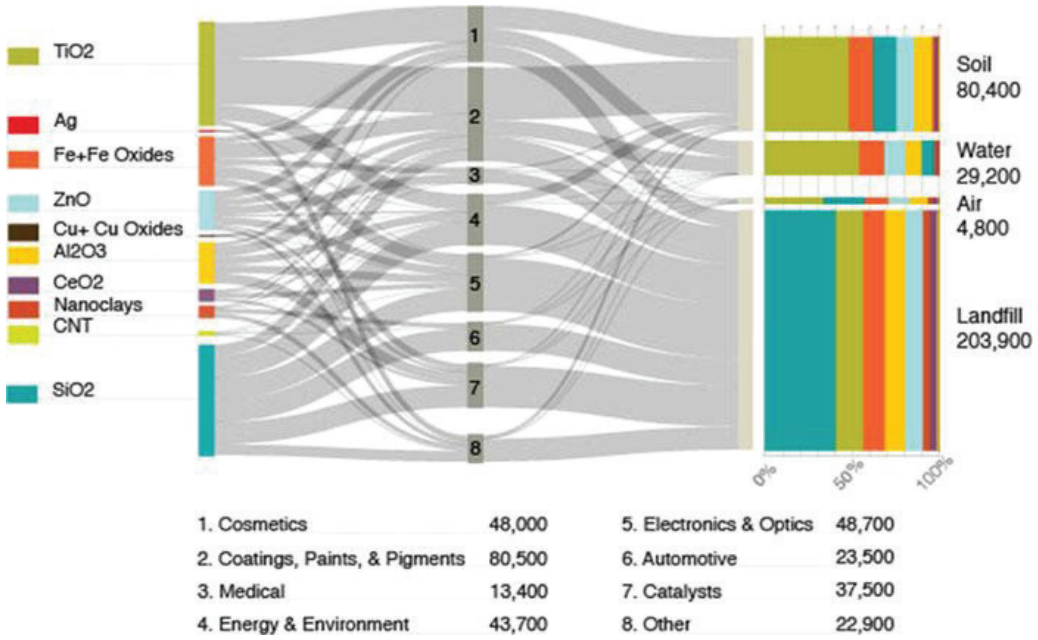


Figure 6: Flux global des nanomatériaux en 2010 (en tonne par an) depuis leur production à leur utilisation et jusqu'à leur éventuelle élimination ou rejet dans l'environnement. Les différents stades du cycle de vie sont représentés de gauche (production) à droite (élimination ou rejet) (Keller *et al.,* 2014).

Les nanomatériaux peuvent atteindre les sols via les eaux usées utilisées pour l'irrigation et surtout à travers l'épandage de boues de stations d'épuration utilisées comme fertilisants des champs agricoles dans certains pays, dont la France (Kiser *et al.*, 2009; Sun *et al.*, 2014; Yang *et al.*, 2014). En effet, il est estimé que 90% des NPs d'argent (Ag), de ZnO, TiO_2 et de cérium (Ce) sont retenues dans les boues lors du retraitement des eaux usées en station d'épuration (Cornelis *et al.,* 2014). Les applications directes de nano-produits sous forme de nanofertilisants ou nanopesticides sont encore très peu prises en compte dans les estimations, du fait de leur apparition récente sur le marché. De plus, l'estimation de la production mondiale de nanomatériaux est encore mal évaluée (Piccinno *et al.*, 2012). Il est donc très probable que les concentrations prédites par ces modèles sous-estiment encore fortement les concentrations réelles dans certains sols agricoles.

2. Devenir des nanomatériaux dans les sols

a. Transformations des nanomatériaux dans les sols

Le fort ratio aire de surface / volume des nanomatériaux entraîne une forte réactivité et des propriétés physico-chimiques très dynamiques dans un contexte environnemental (Lowry *et al.*, 2012). De ce fait, ces polluants émergents peuvent subir de nombreuses transformations lorsqu'ils sont en contact avec les composés et les organismes du sol. Ces transformations sont susceptibles d'affecter leur devenir, leur mobilité et leur toxicité dans les sols (Pan and Xing, 2012). Ces aspects doivent donc être étudiés afin d'améliorer l'évaluation des risques associés à ces contaminants.

Les nanomatériaux peuvent subir 4 grands types de transformations dans l'environnement (Figure 7) : (i) chimiques, (ii) physiques, (iii) biologiques, et (iv) des interactions avec des macromolécules. Ces différents processus peuvent conduire à des modifications d'agrégation (homo- et hétéroagrégation), à la dissolution de certains nanomatériaux et au vieillissement des enrobages (coating) qui peuvent leur être associés.

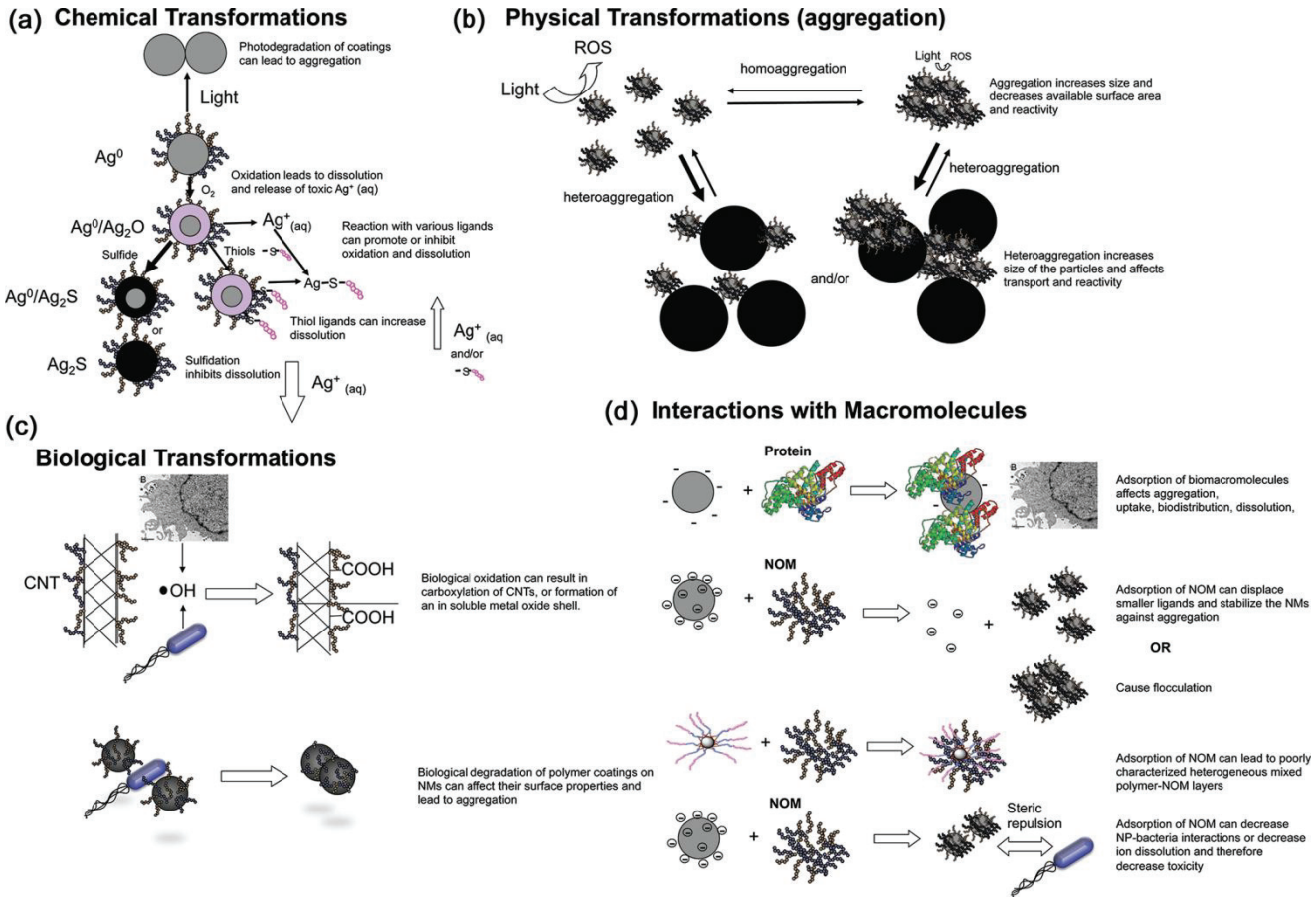


Figure 7: Schéma de synthèse représentant les transformations chimiques (a), physiques (b), biologiques (c) et les interactions avec les macromolécules que les nanomatériaux subissent dans l'environnement, NOM : Natural Organic Matter, ROS : Reactive Oxygen Species, CNT : Carbon Nanotube (Lowry *et al.*, 2012).

En raison de limitations techniques, les transformations des nanomatériaux ne peuvent pas être étudiées directement dans les matrices environnementales complexes comme les sols. Actuellement, les techniques disponibles pour caractériser les nanomatériaux sur la base de leur taille, de leur charge et de leur composition chimique ne sont applicables qu'en phase aqueuse (Dynamic Light Scattering, microscopie électronique à transmission et balayage, microscope à force atomique, EXAFS…) (Tourinho *et al.*, 2012). C'est pourquoi les données disponibles dans la littérature ont été majoritairement acquises à partir de milieux aqueux simplifiés dans lesquels des paramètres physico-chimiques environnementaux d'intérêt ont été modifiés.

Plusieurs facteurs environnementaux sont bien connus pour modifier l'homoagrégation (agrégation entre nanomatériaux, Figure 7b), les plus étudiés étant le pH et la force ionique (Cornelis *et al.*, 2014). En particulier, ces facteurs influencent les charges de surface qui

peuvent être déterminées via la mesure du potentiel zêta (mobilité électrophorétique). Une forte homoagrégation est observée au pH proche du point isoélectrique du nanomatériau (i.e. au pH pour lequel la charge de surface nette est égale à zéro) et également pour une force ionique élevée jusqu'à la concentration critique de coagulation (CCC). Dans ce cas, l'agrégation n'augmente plus à cause de la diffusion (Darlington *et al.*, 2009; Thio *et al.*, 2011; Cornelis *et al.*, 2014). Au vu des valeurs de force ionique retrouvées dans les sols, très en deçà des CCC mesurées, il est prédit que les nanomatériaux aient un faible taux d'homoagrégation dans les sols, excepté aux pH proches du point isoélectrique des NPs considérées (Cornelis *et al.,* 2014).

Le processus physique susceptible d'être le plus courant dans les pores du sol est l'hétéroagrégation (i.e. agrégation avec des colloïdes naturels autres que les nanomatériaux) (Figure 7b et 7d, Cornelis *et al.*, 2011; Zhou *et al.*, 2012). Cette hypothèse est supportée par le fait que les hétéroagrégats de colloïdes naturels sont beaucoup plus fréquents dans les sols que les homoagrégats (Buffle *et al.*, 1998) et que la concentration en particules environnementales est beaucoup plus grande que celle des nanomatériaux. L'hétéroagrégation est un phénomène beaucoup plus difficile à étudier expérimentalement que l'homoagrégation, particulièrement dans les sols car il existe une très grande variété de colloïdes ou particules (matière organique, argile, limon…) pouvant potentiellement interagir avec les nanomatériaux. Des études pionnières se sont intéressées aux processus d'hétéroagrégation et ont montré que la matière organique (MO) dissoute pouvait s'adsorber à la surface des TiO_2-NPs leur conférant un potentiel zêta négatif (Zhang *et al.*, 2009). En milieu aquatique, les argiles de type montmorillonite entraînent une déstabilisation des NPs en suspension, qu'elles soient chargées positivement ou négativement (Ag et TiO_2) et les interactions NPs-argile sont dépendantes du pH et de la force ionique (Zhou *et al.*, 2012). Cornelis *et al., (*2011) ont montré que du phosphate dissous pouvait s'adsorber en surface de CeO_2-NPs, mais aussi qu'une hétéroagrégation pouvait avoir lieu avec des argiles dans des conditions représentatives du sol. Dans une étude associée, les Ag-NPs chargées négativement s'adsorbaient préférentiellement sur les sites de surface en bordure des feuillets d'argile (oxyde de fer ou d'aluminium) qui sont chargés positivement (Cornelis *et al.,* 2011). Les transformations subies par les nanomatériaux dans les sols demeurent néanmoins très difficilement prévisibles. De plus,

elles ne peuvent être généralisées sur la base de ce qui est connu pour un sol donné en raison des caractéristiques propres aux différents types d'argiles, de la typologie des MO, des conditions de pH et de la force ionique qui varient d'un sol à un autre.

En plus des processus d'homo- et hétéroagrégation, certains nanomatériaux peuvent aussi subir une dissolution (Figure 7a). Les nanomatériaux constitués de métaux de classe B (e.g. Ag, Zn) sont plus susceptibles de se solubiliser dans l'environnement car ils forment des oxydes métalliques partiellement solubles et ont une forte affinité avec les ligands inorganiques et organiques sulfurés (Lowry *et al.*, 2012). La dissolution des nanomatériaux entraîne le relargage d'ions métalliques connus pour être toxiques vis à vis des organismes du sol. La compréhension de cette toxicité requiert la connaissance du taux de dissolution dans les sols et de la concentration relative des formes dissoutes et nanoparticulaires au cours du temps (Tourinho *et al.*, 2012). Toutefois, ces données sont encore très limitées à cause des difficultés techniques pour séparer les fractions dissoutes et nanoparticulaires à partir des sols. Vittori Antisari *et al.*, (2013) ont observé une faible dissolution des CeO_2, Fe_3O_4 et SnO_2-NPs dans un sol de texture équilibrée. Dans 16 sols différents, aucune dissolution des CeO_2-NPs n'a été constatée (Cornelis *et al.,* 2011). En revanche, des Ag et ZnO-NPs peuvent se solubiliser de manière significative (Kool *et al.*, 2011; Shoults-Wilson *et al.*, 2011; Rousk *et al.*, 2012; Diez-Ortiz *et al.*, 2015), mais ce processus est limité par la présence d'un enrobage (Coutris *et al.*, 2012). La dissolution des NPs semblant être un processus qui s'effectue sur le long terme dans les sols, il serait nécessaire d'étudier le vieillissement des NPs sur des longues durées et d'évaluer leur toxicité dans ces conditions (Coutris *et al.*, 2012; Whitley *et al.*, 2013; Diez-Ortiz *et al.*, 2015).

La modification des nanomatériaux suite aux activités biologiques est inévitable dans les sols en raison de la production de métabolites par les plantes et les microorganismes ou à l'ingestion par la faune. Ces transformations, qui concernent les nanomatériaux purs ou enrobés, sont notamment associées aux réactions redox en lien avec l'activité biologique (Lowry *et al.,* 2012). Il est vraisemblable que la biotransformation la plus courante soit l'adsorption de biomacromolécules (e.g. protéines, polysaccharides) à la surface des nanomatériaux (forme d'hétéroagrégation, Figure 7c). La biodégradation des nanomatériaux à base de carbone est possible mais dépend du type de sol et des enzymes disponibles dans les pores du sol (Petersen *et al.*, 2011). Il a également été montré que les enrobages des

nanomatériaux utilisés pour limiter leur homoagrégation et favoriser leur stabilité, comme le citrate, peuvent être facilement dégradés, en particulier par des espèces réactives de l'oxygène (Reactive Oxygen Species – ROS) produites par des champignons (Navarro *et al.*, 2011).

Les nanomatériaux peuvent donc subir des transformations multiples et complexes dans les sols qui vont modifier leur agrégation, leur charge de surface et leur réactivité et donc possiblement affecter leur devenir et toxicité. Face à cette complexité, des tentatives sont faites pour classer les différents types de transformations subies par les NPs afin de limiter le nombre d'expérimentations à réaliser et permettre une meilleure évaluation des risques (Mitrano *et al.*, 2015, Figure 8).

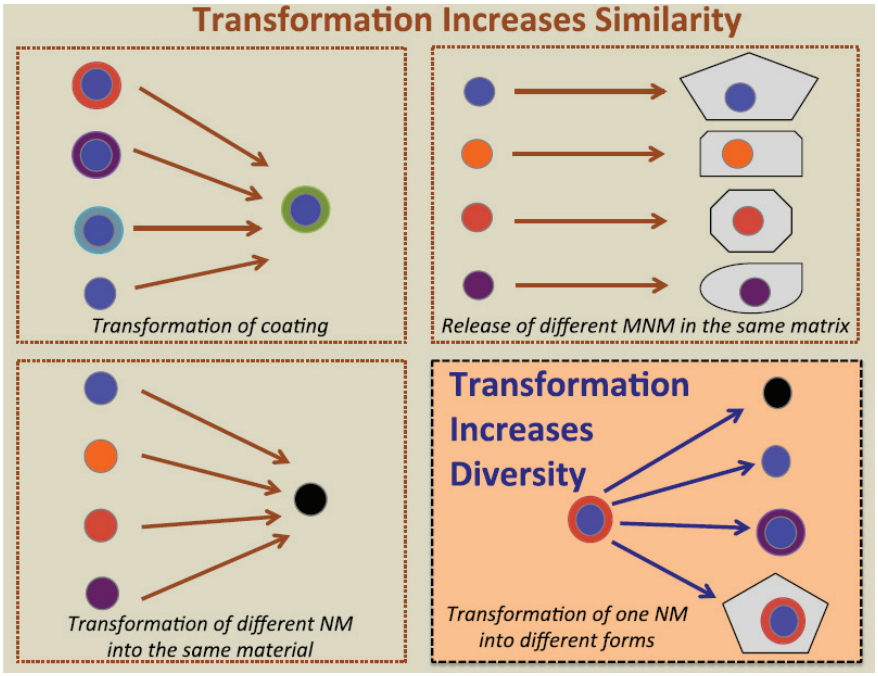


Figure 8: Schéma conceptuel représentant les transformations pouvant augmenter la similarité entre nanomatériaux ou leur diversité de forme, MNM : Manufactured Nanomaterial (Mitrano *et al.*, 2015).

b. Transport des nanomatériaux dans les sols

Depuis une dizaine d'années, un grand nombre d'études a été consacré au transport des nanomatériaux en milieu poreux afin de prédire leur devenir dans les eaux souterraines et les sols. La majorité de ces travaux a été conduite dans des situations simplifiées en utilisant des colonnes représentant des structures porales homogènes (sable ou billes de verre) en

conditions de saturation en eau. Cette littérature se base sur les bonnes connaissances du transport des colloïdes en milieu poreux (e.g. Vitorge, 2010; Elimelech *et al.*, 2013) associées en particulier à la théorie Derjaguin-Landau-Verwey-Overbeek (DLVO). Les différents processus déterminant le transport des colloïdes et des nanomatériaux en milieu poreux sont représentés dans la figure 8.

En utilisant des systèmes simplifiés, plusieurs facteurs environnementaux affectant la mobilité des nanomatériaux ont été identifiés, en particulier la force ionique de la solution, le pH, la présence d'acides organiques, la concentration et la taille des nanomatériaux ou la vitesse de l'eau (Lecoanet *et al.*, 2004; Espinasse *et al.*, 2007; Darlington *et al.*, 2009; Solovitch *et al.*, 2010; Wang *et al.*, 2012). Le transport des nanomatériaux augmente fortement lorsque ceux-ci sont stables en suspension (i.e. ne sédimentent pas) (Wang *et al.*, 2012). Ces conditions sont réunies lorsqu'ils sont faiblement agrégés (taille < 300 nm), que la force ionique de la solution est faible, en présence d'acides humiques ou fulviques, ou de surfactants. La vitesse de l'eau est également à prendre en compte : généralement, plus le flux d'eau est important, plus le transport de NPs est important (Lecoanet and Wiesner, 2004; Chowdhury *et al.*, 2012; Jeong and Kim, 2009). De manière générale, il a été observé que la taille des nanomatériaux augmentait suite à leur transport dans la colonne suggérant des processus d'homo- et d'hétéroagrégation (Figure 9 – processus 3 et 6, (Fang *et al.*, 2009; Wang *et al.*, 2012). Logiquement, les nanomatériaux de plus petite taille ont des capacités de transport plus grandes (Darlington *et al.*, 2009; Ben-Moshe *et al.*, 2010). Darlington *et al.,* (2009) ont montré que les NPs de 200nm avaient une mobilité beaucoup plus importante que les agrégats de plus grande taille due au processus de filtration et à la sédimentation (Figure 9 – processus 5 et 8). Différentes études ont mis en évidence que la mobilité des NPs variait fortement d'un nanomatériau à un autre (Lecoanet and Wiesner, 2004; Lecoanet *et al.*, 2004; Ben-Moshe *et al.*, 2010; Tian *et al.*, 2010). Les nanomatériaux à base de carbone (fullerène, SWCNT, MWCNT) semblent particulièrement peu mobiles comparés aux nanomatériaux métalliques, sans doute à cause de leur très forte hydrophobicité qui empêche leur dispersion dans l'eau et donc leur mobilité (Jaisi *et al.*, 2008; Tian *et al.*, 2012).

Ces données obtenues en conditions simplifiées apportent des informations importantes pour la compréhension du devenir des nanomatériaux dans les sols. Cependant, elles ne représentent pas de manière satisfaisante la variété des types de surfaces minérales et

organiques, l'hétérogénéité des charges de surface, la granulométrie et la chimie de la solution des sols (Fang *et al.*, 2009).

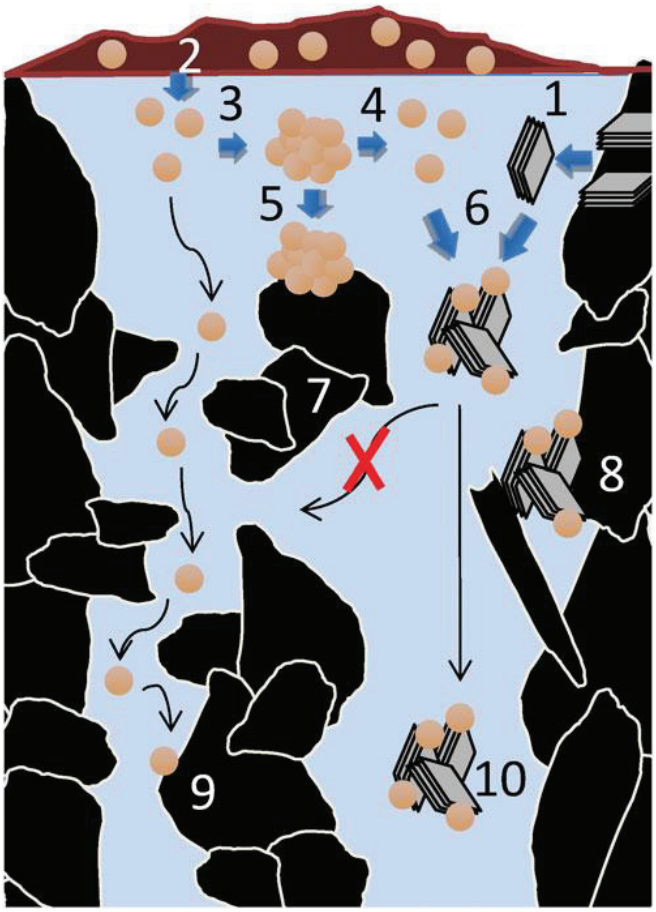


Figure 9: Schématisation des processus clés déterminant le transport des colloïdes et nanomatériaux en milieu poreux: 1. Génération de colloïdes. 2. Lessivage des nanomatériaux. 3. Homoagrégation. 4. Dispersion. 5. Sédimentation. 6. Hétéroagrégation. 7. Exclusion de taille. 8. Filtration (*Straining*). 9. Déposition. 10. Transport convectif (Cornelis *et al.,* 2014).

Un nombre limité de publications concernant le transfert des nanomatériaux dans les sols naturels est disponible à l'heure actuelle (Sun *et al.*, 2015). Il a été montré que les ZnO-NPs ont une mobilité plus faible dans un sol que dans du sable. Par ailleurs, les effets de la concentration de NPs et de la force ionique étaient très différents dans ces 2 types de milieux. Dans le sable, le transport du ZnO diminuait lorsque la concentration appliquée augmentait, alors que le phénomène inverse était observé dans le sol (Sun *et al.,* 2015). Deux études portant sur le transport des TiO_2-NPs ont montré, soit aucun transport dans les 3 sols testés (Nickel *et al.*, 2015), soit une mobilité dans 8 sols sur les 12 étudiés (Fang *et al.*, 2009). Dans cette dernière étude, le transport du TiO_2 était influencé par la teneur en argile et la force ionique de la solution du sol. Dans ces 2 études, les concentrations, la préparation

des colonnes et les protocoles de minéralisation étaient très différents, ce qui a pu influer de manière importante sur les résultats obtenus. Nickel *et al.,* (2015) ont tenté d'appliquer les protocoles standardisés de l'OCDE TG 312 « Lessivage en colonne de sol » et suggèrent que ce protocole doit être adapté lorsque l'on utilise des nanomatériaux, en particulier le TiO_2.

Un transport limité des nanomatériaux à base de carbone dans les sols (fullerènes, SWCNT et MWCNT) a également été rapporté (Jaisi and Elimelech, 2009; Kasel *et al.*, 2013). Le même constat a été fait pour les quantums dots de CdSe et CdSe/ZnS, dont la majorité était retenue à l'entrée de la colonne (Navarro *et al.*, 2011). Ces auteurs ont montré toutefois que la mobilité des quantums dots était significativement augmentée en présence d'acides organiques au pouvoir chélatant (comme l'EDTA), ces derniers pouvant être présents en quantité importante dans les sols, notamment via l'exsudation racinaire. Cornelis *et al.,* (2013) ont observé le transport d'Ag-NPs dans 8 sols sur les 11 testés, en appliquant une concentration très faible de 1.7 mg Ag L^{-1}. Le transfert des Ag-NPs était cependant assez faible (91 à 98.8% retenus dans le sol), alors que les sols utilisés étaient très sableux (60 à 99% de sable). La faible mobilité des Ag-NPs a été expliquée par une forte hétéroagrégation entraînant des processus d'exclusion de taille, de filtration et de déposition sur des oxalates d'aluminium positivement chargés (Cornelis *et al.*, 2013, Figure 10, Figure 9 –processus 7, 8 et 9).

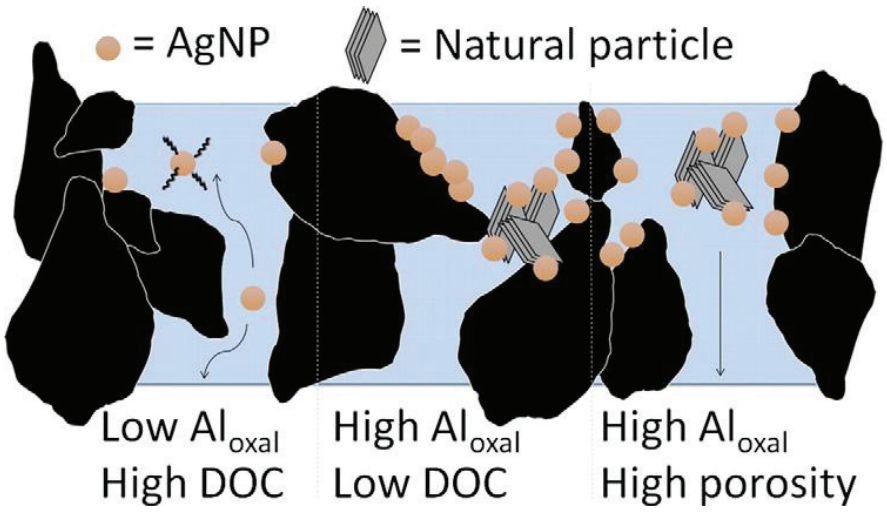


Figure 10: Influence de la concentration en oxalates d'aluminium et en carbone organique dissout sur la mobilité des Ag-NPs dans les sols (Cornelis *et al.,* 2013)

Toutes, ces études pionnières suggèrent que les nanomatériaux ont une très faible mobilité dans les sols naturels et donc qu'il existe un faible risque de transport de ces contaminants

hors du site pollué, que ce soit verticalement vers les eaux souterraines ou latéralement vers des sites voisins. Cependant, ces conclusions restent à valider en raison du faible nombre d'études disponibles (6 types de NPs étudiés pour 12 publications au total) et des nombreuses questions qui subsistent. En effet, la plupart des études disponibles ont été conduites en conditions de saturation en eau, avec de fortes concentrations en nanomatériaux, des méthodologies variées en termes de préparation des suspensions de nanomatériaux et en utilisant principalement des sols de type sableux. A l'heure actuelle, les paramètres clés du sol influençant le transport des nanomatériaux ne sont pas identifiés, alors que ces informations permettraient une meilleure évaluation des risques suite à des épandages de boues ou l'utilisation de nanofertilisants sur un sol. De plus, il apparait nécessaire d'étudier la mobilité des nanomatériaux dans des conditions plus réalistes d'exposition en considérant par exemple des apports via l'épandage de boues, des contaminations chroniques, l'application de faibles concentrations ou l'influence de la végétation.

3. Biodisponibilité et toxicité des nanomatériaux dans les sols

a. Biodisponibilité des nanomatériaux dans les sols

Les nanomatériaux peuvent avoir des temps de résidence longs dans les sols et subir de nombreuses transformations. Les plantes et les organismes du sol (microorganismes, mésofaune et macrofaune) seront donc exposés à ces contaminants émergents. Dans ce contexte, le point clé est de déterminer comment les organismes sont exposés aux nanomatériaux présents dans les différents compartiments du sol, qu'il s'agisse de la solution du sol ou des agrégats et quelle est la fraction biodisponible de ces contaminants en fonction des organismes étudiés (Tourinho *et al.*, 2012). Selon la définition de Semple *et al.,* (2004), la biodisponibilité d'un composé dans les sols est définie de la manière suivante en écotoxicologie : « *un composé biodisponible est librement disponible pour traverser la membrane cellulaire d'un organisme à partir du milieu où l'organisme se trouve à un temps donné* » et la bioaccessibilité est définie ainsi : *« le composé bioaccessible est librement disponible pour traverser la membrane cellulaire d'un organisme depuis l'environnement, si l'organisme a accès à ce composé. Toutefois, le composé peut être soit éliminé physiquement de l'organisme ou seulement biodisponible après un certain temps*". D'après ces définitions,

la bioaccessibilité englobe donc ce qui est actuellement biodisponible et ce qui est susceptible de le devenir (Figure 11, Semple *et al.*, 2004). Dans les sols, on s'attend donc à ce que la concentration biodisponible en nanomatériaux soit plus faible que la concentration totale, du fait du grand nombre de surfaces réactives pouvant immobiliser les nanomatériaux et de transformations pouvant affecter leur biodisponibilité (Cornelis *et al.,* 2014).

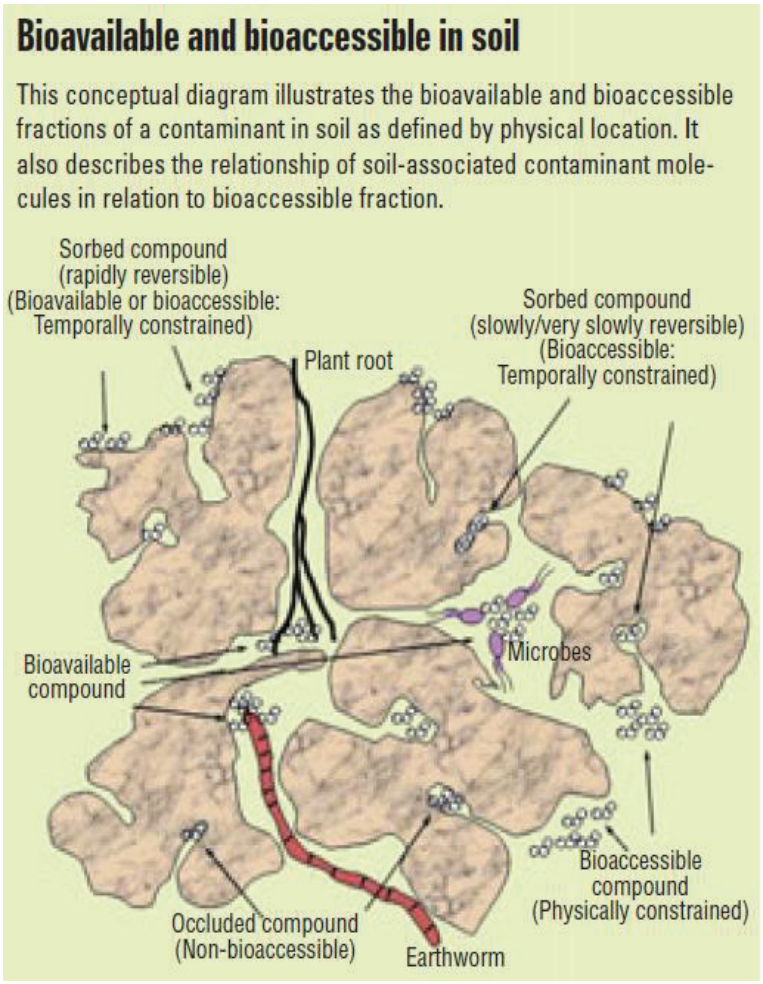


Figure 11: Biodisponibilité et bioaccessibilité des contaminants dans les sols (Semple *et al.,* 2004).

Aujourd'hui, nous ne sommes pas capables de mesurer la concentration totale d'un nanomatériau dans un sol en raison de limitations techniques. Evaluer la fraction biodisponible et bioaccessible des nanomatériaux est un défi encore plus grand qui nécessite des développements méthodologiques importants et donc les données à ce sujet sont quasi-inexistantes (Vittori Antisari *et al.*, 2013). Toutefois, lorsque la toxicité des nanomatériaux repose sur la dissolution de métaux comme dans le cas de l'Ag ou du Zn, les études peuvent se baser sur des méthodes bien connues pour évaluer la fraction biodisponible des éléments

traces dissous (Cornelis *et al.,* 2014). Même si nous ne pouvons pas mesurer la fraction biodisponible, de nombreuses études ont montré que les nanomatériaux étaient retrouvés dans les racines ou les feuilles de différentes plantes (Rico *et al.*, 2011), dans les tissus d'invertébrés (Tourinho *et al.,* 2012) ou dans des champignons mycorhiziens (Whiteside *et al.*, 2009), illustrant leur biodisponibilité réelle dans les sols. Toutefois, il faut rester prudent, un composé biodisponible n'est pas nécessairement toxique, selon la concentration présente et les propriétés du polluant dans l'environnement considéré.

b. Toxicité des nanomatériaux dans les sols

De nombreuses études *in vit*ro ont montré la toxicité des nanomatériaux et identifié des mécanismes de toxicité (e.g. Nel et al. 2006). Les principaux mécanismes invoqués reposent principalement sur l'adsorption de NPs à la surface des cellules, leur dissolution et des stress oxydatifs induits par la production de ROS (H_2O_2, $OH^{\cdot}$, $O_2^{\cdot -}$, $^1O_2$) (Figure 12 ; (Nel *et al.*, 2006; Handy *et al.*, 2008; Jiang *et al.*, 2008; Simon-Deckers *et al.*, 2009; Li *et al.*, 2012). De nombreux effets au niveau des membranes bactériennes ont été rapportés, comme la dépolarisation ou l'augmentation de la perméabilité ainsi que des dommages au niveau de protéines ou de l'ADN ((Jomini *et al.*, 2012; Sohm *et al.*, 2015; Bondarenko *et al.*, 2012).

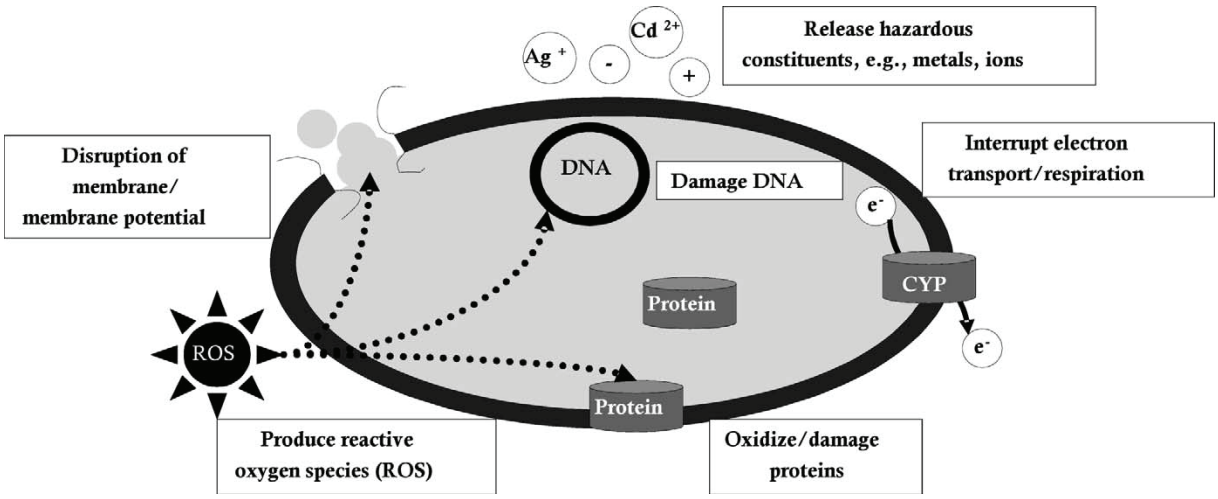


Figure 12: Schématisation des possibles mécanismes de toxicité des nanomatériaux sur des cellules bactériennes. CYP=cytochrome P (Handy *et al.,* 2008)

Toutefois, il est difficile de savoir si ces mécanismes de toxicité observés *in vitro* seront similaires en conditions environnementales. En effet, à partir des connaissances disponibles sur les transformations des nanomatériaux dans les sols (Partie 2. a.), on s'attend à ce que

les facteurs abiotiques influencent les propriétés physico-chimiques et donc la toxicité de ces contaminants. Un paramètre sans doute clé pour leur toxicité est l'agrégation, car elle réduit l'aire de surface « disponible » et donc leur réactivité (Hotze *et al.*, 2010). L'agrégation pourrait donc réduire leur toxicité lorsqu'elle est associée à des processus de surface, comme l'émission de ROS et la dissolution (Lowry *et al.,* 2012). D'autre part, l'hétéroagrégation entraîne une augmentation de la taille effective des nanomatériaux, ce qui pourrait limiter leur ingestion par certains organismes ou le passage à travers une paroi et/ou membrane cellulaire (Tourinho *et al.,* 2012). Ainsi certains paramètres physico-chimiques du sol identifiés pour favoriser ou limiter l'hétéroagrégation pourraient être de bons indicateurs de la biodisponibilité des nanomatériaux. Une force ionique élevée et la présence d'argile semblent être des conditions favorables à une forte hétéroagrégation des NPs et conduirait donc à de faibles biodisponibilité et toxicité (Shoults-Wilson *et al.*, 2011). Cependant en présence de MO dissoute, il a été observé une augmentation de l'écotoxicité/accumulation associée à une augmentation de la mobilité dans les sols (Cornelis *et al.,* 2014). De ce fait l'hétéroagrégation n'implique pas forcément une diminution de la biodisponibilité et de la toxicité des nanomatériaux dans les sols.

L'évaluation de la biodisponibilité et de la toxicité des nanomatériaux et des facteurs du sol les influençant en est encore à ses balbutiements, mais quelques directions à suivre pour les études futures peuvent d'ores et déjà être identifiées. La première est l'amélioration des méthodologies pour caractériser les nanomatériaux dans des conditions environnementales proches du sol et le développement des protocoles permettant d'évaluer la fraction biodisponible/bioaccessible des NPs en milieux hétérogènes complexes. Le second point est l'évaluation de l'influence des propriétés du sol, telles que la texture (teneur en argile, limon, sable), de la force ionique et de la teneur en MO sur l'écotoxicité des nanomatériaux, car il semble probable que ces facteurs affecteront leur biodisponibilité et toxicité.

4. Impact des nanomatériaux sur les communautés microbiennes du sol

L'écotoxicité des nanomatériaux dans les sols peut être évaluée en s'intéressant à leur impact sur différents organismes et/ou communautés d'organismes retrouvés dans les sols (e.g. microorganismes, vers de terre, nématodes). Parmi eux, les communautés microbiennes, à savoir les bactéries, les archées et les champignons, sont reconnues comme étant des indicateurs pertinents et sensibles pour l'évaluation de l'impact des polluants sur le fonctionnement biologique du sol (Kandeler *et al.*, 1996; Giller *et al.*, 1998; Schloter *et al.*, 2003; Holden *et al.*, 2014). En effet, du fait de leur rôle dans les cycles biogéochimiques, les communautés microbiennes sont impliquées dans des services écosystémiques associés à la fertilité et la production végétale. Les microorganismes ont également un rôle clé dans la dégradation des polluants dans les sols (Leahy and Colwell, 1990).

La réponse des communautés microbiennes à des perturbations environnementales et notamment aux stress chimiques, peut être appréhendée en évaluant de façon complémentaire les effets sur l'activité, l'abondance et la diversité des communautés microbiennes (Griffiths and Philippot, 2013). Une synthèse bibliographique dédiée spécifiquement aux effets des NPs sur les communautés microbiennes de sol a été rédigée.

a. « Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review"

L'article 1 intitulé « Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review" a été publié en 2015 dans le Journal *Environmental Science and Pollution Research* dans le cadre d'un numéro spécial *Microbial Ecology of the Terrestrial and Coastal Environments*.

Environ Sci Pollut Res
DOI 10.1007/s11356-015-4171-x

MICROBIAL ECOLOGY OF THE CONTINENTAL AND COASTAL ENVIRONMENTS

Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review

Marie Simonin · Agnès Richaume

Received: 24 October 2014 / Accepted: 22 January 2015

Abstract This report presents an exhaustive literature review of the effects of engineered nanoparticles on soil microbial communities. The toxic effects on microbial communities are highly dependent on the type of nanoparticles considered. Inorganic nanoparticles (metal and metal oxide) seem to have a greater toxic potential than organic nanoparticles (fullerenes and carbon nanotubes) on soil microorganisms. Detrimental effects of metal and metal oxide nanoparticles on microbial activity, abundance, and diversity have been demonstrated, even for very low concentrations (<1 mg kg^{-1}). On the opposite, the negative effects of carbon nanoparticles are observed only in presence of high concentrations (>250 mg kg^{-1}), representing a worst case scenario. Considering that most of the available literature has analyzed the impact of an acute contamination of nanoparticles using high concentrations in a single soil, several research needs have been identified, and new directions have been proposed. The effects of realistic concentrations of nanoparticles based on the concentrations predicted in modelization studies and chronic contaminations should be simulated. The influence of soil properties on the nanoparticle toxicity is still unknown and that is why it is crucial to consider the ecotoxicity of nanoparticles in a range of different soils. The identification of soil parameters controlling the bioavailability and toxicity of nanoparticles is fundamental for a better environmental risk assessment.

Keywords Nanomaterials · Microbial ecotoxicology · Terrestrial ecosystem · Soil pollution · Risk assessment · Nanoscale zero valent iron

Responsible editor: Philippe Garrigues

M. Simonin · A. Richaume
Université de Lyon, Lyon, France

M. Simonin · A. Richaume
Université Claude Bernard Lyon 1, Villeurbanne, France

M. Simonin · A. Richaume (✉)
Microbial Ecology, CNRS, UMR 5557, Université Lyon 1, Villeurbanne, France
e-mail: agnes.richaume@univ-lyon1.fr

M. Simonin
LTHE, UMR 5564, UJF-Grenoble/CNRS-INSU/G-INP/IRD, Grenoble F-38041, France

Published online: 04 February 2015

Introduction

Because of their unique properties, engineered nanoparticles (NPs) are increasingly produced and used in diverse commercial products (agriculture, cosmetics, energy, electronics, paint, medicine…). Toxiceffects of NPs have been observed in numerous in vitro studies; in particular, many NPs, such as silver-, copper-, or zinc oxides-NPs, are known to have antimicrobial properties (Neal 2008; Fabrega et al. 2009; Dinesh et al. 2012). The toxicity mechanism often mentioned is an oxidative stress generated by the production of reactive oxygen species (ROS) from NPs in contact with microbial membranes, causing disruption of membranes, oxidation of proteins, or interruption of energy transduction (Klaine et al. 2008; Neal 2008; Xia et al. 2008).

During the different phases of their life cycle, from production to disposal, NPs can be released to the environment, raising great concerns about potential ecological risks. Direct measurement of NP concentration in the environment remains difficult due to current technical limitations (Cornelis et al. 2014). Currently, the only way to get information on existing levels of NPs in the environment is to model predicted environmental concentrations (Sun et al. 2014). Exposure modeling strongly suggests that soil could be a major sink of NPs

compared to air and water ecosystems (Gottschalk et al. 2009; Keller et al. 2013; Sun et al. 2014).

NPs can enter soil through various pathways, such as agricultural amendments of sewage sludge, atmospheric deposition, landfills, or accidental spills during industrial production. The current models estimate that in sewage sludge-treated soil, TiO_2-NP concentrations increase between 0.94 and 3.6 mg kg^{-1} per year, whereas for Ag-NPs and fullerenes, the yearly predicted increases are more than 1000-fold lower. In this case, concentration increases are between 0.09 and 0.65 μg kg^{-1} and between 0.38 and 1.5 μg kg^{-1}, respectively (Sun et al. 2014). Intentional applications of NPs can also be possible in a context of soil remediation. Nanoscale zero-valent iron (nZVI) particles have the potential to remediate diverse environmental contaminants such as chlorinated organic compounds or inorganic compounds for example. This remediation strategy has been mainly employed for the decontamination of groundwater, and its utility in soil remediation is being increasingly considered (Naja et al. 2009; Satapanajaru et al. 2008). Therefore, there is a demand to assess the risks associated to NPs in soils, in order to preserve the soil capacity to fulfill essential ecosystem services.

Soil microbial communities are both relevant and sensitive indicators of soil perturbations (Brookes 1995; Kandeler et al. 1996; Holden et al. 2014) because of their key role in biogeochemical cycling (carbon, nitrogen, phosphorous, sulfur cycles), biodegradation of pollutants, crop production, and climate. A microbial ecotoxicological approach based on the response of soil microbial communities offers the opportunity to evaluate the impact of pollutants on natural assemblages of populations, contrary to toxicological studies which use single populations in synthetic media. This approach allows a realistic assessment of the response of natural communities to NP contamination and leads consequently to a better environmental risk assessment. Environmental constraints and/or anthropogenic perturbation can trigger a microbial response observable through different variables among the community. The microbial community responses to a disturbance can be monitored using (i) activity rates, (ii) abundances, and (iii) diversity (Brookes 1995; Griffiths and Philippot 2013). Through this review, we provide a synthesis of studies assessing the overall impact of NPs on soil biodiversity and functioning using the descriptors mentioned above.

Among NPs, a wide range of different materials with diverse physical, chemical, and toxicological properties exist. Inorganic and organic NPs can be distinguished based on their core material (Ju-Nam and Lead 2008). Inorganic NPs are divided into metal, metal oxide, and quantum dots NPs, while fullerenes and carbon nanotubes (single and multi-walled CNTs) are defined as organic NPs. Since NPs cannot be considered as a single homogeneous group of contaminants, we will review separately the effects of (i) metal and metal oxides, (ii) carbon nanoparticles, and (iii) nZVI. Due to increasing interest of this latter for in situ soil remediation, a specific attention will be addressed to the nZVI. The direct application to soils of large amounts of nZVI for remediation purposes raise specific concerns about potential consequences on soil microbial communities and their key functions for soil fertility and biodegradation of pollutants.

Impact of nanoparticles on microbial activities

Soil microbial activities are good indicators of soil quality, since soil microorganisms control the transformation and mineralization of natural compounds and xenobiotics (Schloter et al. 2003). The impact of perturbations on microbial activity is classically approached through measurements of "broad" processes (Schimel and Schaeffer 2012) of soil respiration or generalist enzyme activities such as β-glucosidase, urease, and dehydrogenase. Some specific transformations or "narrow" processes realized by a phylogenetically constrained group of microorganisms (Schimel and Schaeffer 2012) can be also analyzed, such as nitrification in nitrogen cycle or the ability to degrade specific pollutants.

Impact of metal and metal oxide nanoparticles

The use of microbial activities to assess the effect of metal or metal oxide NPs is often encountered in the literature (Table 1). Contrasted responses can be observed according to the type of NPs, the concentration, the exposure time, and the measured activity.

Ag-NP has been found to reduce substrate-induced respiration and enzymatic activities after 50 days of exposure to a low concentration of Ag-NPs (0.14 mg kg^{-1} soil) applied in mesocosms via sewage sludge (Colman et al. 2013). Another study using much lower concentrations of Ag-NPs (0.0032, 0.032, 0.32 mg kg^{-1} soil) did not show any changes in the different tested enzymatic activities (Hänsch and Emmerling 2010). However, the authors have found a reduction of the net nitrogen mineralization and an increase of the metabolic quotient suggesting a reduction in substrate use efficiency in presence of Ag-NPs. Shin et al. (2012) have also reported adverse effects of Ag-NPs on enzymatic activities, especially on urease, but the larger reductions were observed only for high concentrations (100 and 1000 mg kg^{-1} soil). Using soluble silver ions as a positive control, these studies show that NPs themselves were responsible for the negative effects on soil microorganisms but not the silver ions dissolved from Ag-NPs (Shin et al. 2012; Colman et al. 2013).

Fe_2O_3-NPs stimulated urease and invertase activities, whereas Fe_3O_4-NPs did not induce any modification of enzymatic activities in presence of high concentrations (420, 840, and 1260 mg kg^{-1} soil) (He et al. 2011). These stimulations

Table 1 Review of the effects of metal and metal oxide nanoparticles on soil microbial communities

Metal and metal oxide nanoparticles					
Reference	NPs	NPs concentrations in soil	Soil	Experiment	Major results
Colman et al. (2013)	Ag-NP	0.14 mg kg^{-1}	Sandy loam (USA)	Mesocosms field experiment-Application of sewage sludge. 0,1 and 50 days	Modification of bacterial community structure (T-RFLP) after 1 day. Stimulation of N_2O fluxes after 8 days. Reduction of SIR and enzymatical activities (phosphatase and leucine aminopeptidase) after 50 days. Larger effect of Ag-NP than the positive control $AgNO_3$.
Hänsch and Emmerling (2010)	Ag-NP	3.2, 32,320 µg kg^{-1}	Sandy-loam (Germany)	Mesocosms– 1, 7,14 and 120 days	Decrease of microbial biomass in a dose-response manner. Increases of basal respiration and metabolic quotients. No effect on microbial biomass N and fluorimetric enzymes.
Peyrot et al. (2014)	Ag-NP	1.25 µg to 30 mg kg^{-1}	Sandy soil with or without compost amendment (Canada)	Microcosms – 6 weeks	Decrease of enzymeactivities (phosphomonoesterase, β-D-glucosidase, arylsulfatase, leucine-aminopeptidase), especially at the low Ag concentrations. No protective role of OM was observed because concentrations of dissolvedAg were similar in both amended and unamended soils.
Shin et al. (2012)	Citrate coated Ag-NP	1, 10, 100 and 1000 mg kg^{-1}	Sandy (Korea)	Microcosms - 1 and7 days	Strong negative dose-response effect on urease activity. Reduction of 5 enzymatic activities in the two highest concentrations(acid phosphatase, arylsulfatase, β-glucosidase, dehydrogenase and fluorescein diacetate hydrolase). The effects of silver ions dissolved from the Ag-NP were not significant, indicating the adverse effectscaused by Ag-NP themselves.
Vittori Antisari et al. (2013)	CeO_2-NP, Fe_3O_4-NP, SnO_2-NP	0, 10 and 100 mg kg^{-1}	Sandy clay loam (Italy)	Microcosms - 7 and 60 days	No effect on C and N microbial biomass. Increased of metabolic quotient (qCO2) in the polluted soil.
Kumar et al. (2011)	Cu-NP, Ag-NP, Si-NP	660 mg kg^{-1}	Sandy -peaty Arctic soil (Canada)	Microcosms - 176 days	Reduction of the diversity of substrate utilization (Biolog) to 16 % in Ag NP-treated soil, and to 40 % and 52 % in Cu and SiO_2 NP-treated soil. No effect on FAME profiles. Ag-NP caused a modification of bacterial composition (DGGE analysis).
Kumar et al. (2012)	Mixture of Cu-NP, Ag-NP and Si-NP	220 mg kg^{-1} soil x 3 NPs=660 mg kg^{-1}	Sandy -peaty Arctic soil (Canada)	Microcosms - 176 days	Reduction of C and N microbial biomass. Modification of microbial community structure (FAME analysis) and especially bacterial community (DGGE analysis).No effect on microbial community level physiological profiles.
Ben-Moshe et al. (2013)	CuO, Fe3O4	10 g kg^{-1}	2 soils: a red sandy clay loam soil and a Rendzina soil (Israel)	Microcosms - 24 or 48 h incubation	NPs affected the soil bacterial community composition, based on DGGE fingerprinting. Both soil types were less affected by Fe3O4 contamination than by CuO contamination, suggesting lower toxicity of Fe_3O_4 to soil bacterial communities.
Rousk et al. (2012)	CuO-NP ZnO-NP	8 concentrations at logarithmic intervals from 0 to 200 mmol $metal.g^{-1}$	2 soils: a «mineral» and an «organic» soil (sandy loam) (UK)	Microcosms - 5-7 h of incubation	CuO-NP decreased bacterial growth (leucine incorporation method) only in the «mineral» soil. NP ZnO reduced bacterial growth in both soil. The toxic effect of these NP may be explained by their dissolved forms.
Collins et al. (2012)	Cu^0-NP, ZnO-NP	Application of 550 mg of NP powder at the center of mesocosms	Agricultural soil (USA)	Field mesocosms- 1, 7, 30 and 162 days	Effect on microbial community level physiological profiles only after 1 day. No effect on FAME profiles. Potential alteration of bacterial community structure (pyrosequencing 454), especially Flavobacteriales and Sphingomonadales.
He et al. (2011)	Fe_3O_4-NP, γ-Fe_2O_3-NP	420, 840 and 1260 mg kg^{-1}	Agricultural soil(China)	Microcosms - 0, 15 and 30 days	No effect on bacterial abundance. Increase of enzymatic activities (urease and invertase) that could be explained by a stimulation of specific groups of Actinobacteria (DGGE analysis).
Ge et al. (2011)	TiO_2-NP, ZnO-NP	For TiO_2: 500, 1000 and 2000 mg kg^{-1} soil For ZnO: 50, 100 and 500 mg kg^{-1} soil	Loam (USA)	Microcosms - 15 and 60 days	Decreases of SIR and microbial biomass (DNA quantity) by both NPs, with differentdose-response effects: linear for TiO_2-NP and exponential for ZnO-NP. No effect on basal respiration. TiO_2-NP

Table 1 (continued)

Metal and metal oxide nanoparticles						
						and ZnO-NP altered soil bacterial community structure and decreased bacterial diversity (T-RFLP analysis).
Ge et al. (2012)	TiO_2-NP, ZnO-NP	For TiO_2: 500, 1000 and 2000 mg kg^{-1} soilFor ZnO: 50, 100 and 500 mg kg^{-1} soil	Loam (USA)	Microcosms - 15 and 60 days	TiO_2-NP and ZnO-NP altered soil bacterial community structure (Pyrosequencing 454) associated to a decrease of 12 taxa: class *Alphaproteobacteria*, the order *Rhizobiales*, families *Bradyrhizobiaceae*, *Geodermatophilaceae*, *Methylobacteriaceae*, *Micromonosporaceae*, and *Rhodospirillaceae*, and the genera *Actinoplanes*, *Balneimonas*, *Blastococcus*, *Bradyrhizobium*, and *Skermanella*. Increase of the abundance of 3 taxa (*Sphingomonadaceae*, *Streptomycetaceae* and *Streptomyces*)known to be associated with the decomposition of recalcitrant organic pollutants and biopolymers. The abundance of these 3 taxa wascorrelated to an increase of protease activity.
Ge et al. (2013)	TiO_2-NP	20 g kg^{-1}	Sandy-clay-loam (USA)	Microcosms -288 days	Decrease of bacterial diversity and modification of bacterial community structure (T-RFLP analysis).
Simonin et al. (2014)	TiO_2-NP	1 and 500 mg kg^{-1}	6 soils : sandy-loam, loam and silty-clay with low or high OM content (France)	Microcosms – 7, 30 and 90 days	Decrease of substrate-induced respiration only in one soil (silty-clay soil with high OM content), even for the low concentration. No effect on bacterial abundance.
Du et al. (2011)	TiO_2-NP, ZnO-NP	TiO_2-NP: 91 mg kg^{-1} soil ZnO-NP: 45 mg kg^{-1} soil	Silty – c lay (China)	Field lysimeters – 9 months	TiO_2-NP: Increase of urease and decreases of catalase and peroxidase. ZnO-NP: Decreases of protease, catalase and peroxidase.
Nogueira et al. (2012)	TiO_2-NP, $TiSiO_4$-NP, CdSe/ZnS quantum dots, gold nanorods, Fe/Co magnetic fluid	TiO_2-NP and$TiSiO_4$-NP: 5 g kg^{-1} soil ;CdSe/ZnS quantum dots and Fe/Co magnetic fluid : 0.5 mg kg^{-1} soil ; gold nanorods : 3.34 mg kg^{-1} soil	Standard artificial OECD soil	Microcosms – 30 days	Higher impact of TiO_2 and gold nanorods in the structural diversity of bacterial community (DGGE analysis). No effect or limited effects of $TiSiO_4$, CdSe/ZnS quantum dots and Fe/Co magnetic fluid NPs on DGGE profiles.
Shah and Belozerova (2009)	Si-NP, Pd -NP, Au-NP, Cu-NP	130 and 660 mg kg^{-1} soil	Soil (USA)+potting mix	Microcosms- 15 days	No effect of the NPs on substrate utilization patterns of microbial communities, nor on FAME profiles.
Shah et al. (2014)	Fe-NP, Co-NP, Ni-NP	Application of 550 mg of NP powder at the center of mesocosms	Agricultural soil (USA)	Field mesocosms - 50 days	No decrease of bacterial richness (Pyrosequencing 454) but some genera were affected by the NPs. *Sphingomonas* and *Lysobacter* genera were decreased, whereas *Flavobacterium* and *Niastella* genera were increased.

were explained as a consequence of changes observed within the bacterial community.

Aged TiO_2-NPs (91 mg kg^{-1} soil) stimulated urease activity but decreased catalase and peroxidase activities after 9 months of incubation in lysimeters (Du et al. 2011). In the same experiment, ZnO-NPs (45 mg kg^{-1} soil) reduced protease, catalase, and peroxidase activities. Ge et al. (2011) also evaluated the impact of TiO_2 and ZnO-NPs on substrate-induced respiration and found a reduction after 15 and 60 days with 500, 1000, and 2000 mg kg^{-1} soil. TiO_2-NPs impact on substrate-induced respiration was also assessed in six contrasted agricultural soils (1 and 500 mg kg^{-1}), and a significant decrease of this activity was noted only in one soil presenting a silty clay texture and a high OM content (Simonin et al. 2014).

These results indicate that metal and metal oxides can induce modifications of microbial activities in soil and consequently on biogeochemical cycles. The decrease of respiration and enzymatic activities in response to very low concentrations of Ag-NPs illustrates the high toxic potential of some metal NPs.

Impact ofcarbon nanoparticles (fullerene and CNTs)

Fullerene (C_{60}) had no adverse effect on soil respiration or enzymatic activities (Table 1, Tong et al. 2007; Johansen et al. 2008) even after 180 days of incubation. In the same range of concentrations, no modification of microbial activities (soil respiration and enzymatic activities) was reported for MWCNT (Shrestha et al. 2013), except for the highest concentrations used (500 and 5000 mg kg^{-1} soil) (Chung et al. 2011). Likewise, SWCNT caused a reduction of the enzymatic activities only at high concentrations (300 to 1000 mg kg^{-1} soil) (Jin et al. 2013).

Taken together, these data suggest that carbon NPs have a very low toxic potential to soil microorganisms compared to metal and metal oxide NPs, because modifications of microbial activities were only observed at very high concentrations (>500 mg kg^{-1} soil) representing the worst case scenario given the present annual production of CNTs (Keller et al. 2013).

Impact of nanoscale zero valent iron

Tilston et al. (2013) performed an experiment with a sandy loam soil artificially contaminated with the PCB Aroclor-1242 to simulate the utilization of nZVI in a context of remediation of organochlorine-contaminated environments. The authors reported a decrease of the activity of chloroaromatic mineralizing microorganisms (2,4-D mineralization), which biodegradative functions could contribute to contaminant remediation. Nevertheless, this negative effect should be taken with caution due to the possible induction of confounding factors as shown by Cullen et al. (2011). No conclusive evidence for negative effects of nZVI was observed due to the redox properties of these particles that induced a significant abiotic modification of the concentration of either the product or substrate of assays for nitrification or deshydrogenase activities. When examining the impact of redox active particles such as nZVI on microbial oxidation–reduction reactions, sterile controls should be made to take into consideration these potential confounding factors (Cullen et al. 2011).

The influence of environmental physicochemical properties on nZVI toxicity and bioavailability was investigated by comparing their impact in different soils. Pawlett et al. (2013) found that microbial respiration induced by multiple substrates was decreased in a clay soil spiked with 270 mg nZVI kg^{-1} soil but not in sandy or loam soils for the same dose.

Impact of nanoparticles on microbial biomass and abundance of functional groups

Microbial biomass is a sensitive indicator of pollution in soils (Brookes 1995). It has been used as an indicator of the impact of NPs in the majority of studies assessing their ecotoxicity. Different methodologies are used in NPs ecotoxicology studies to quantify microbial biomass in soils, such as fumigation–extraction or extractable soil DNA (Hänsch and Emmerling 2010; Ge et al. 2011). In addition to these approaches that assess the total biomass including bacteria, fungi, and archaea, the abundance of specific groups of microorganism targeting universal genes (*16S* or *18S*rRNA genes) or functional genes can be assessed using quantitative PCR (Fajardo et al. 2012).

Impact of metal and metal oxide nanoparticles

Different concentrations of Ag-NPs (0.0032, 0.032, 0.32 mg kg^{-1} soil) induced a decrease in microbial biomass in a dose–response manner (Hänsch and Emmerling 2010). However, soil contamination with iron oxide magnetic NPs (Fe_2O_3 and Fe_3O_4) had no effect on microbial biomass with 10 and 100 mg kg^{-1} soil (Vittori Antisari et al. 2013) nor on bacterial abundance with 420, 840, and 1260 mg kg^{-1} soil (He et al. 2011). Similarly, Simonin et al. (2014) found no significant effect of TiO_2-NPs on bacterial abundances in the six soils tested. TiO_2 and ZnO NPs using 500, 1000, and 2000 mg kg^{-1} soil, both reduced extractable soil DNA (Ge et al. 2011). However, the dose–response curves were linear with TiO_2-NPs and exponential with ZnO-NPs (Ge et al. 2011). These differences were explained by contrasting bioavailability and environmental behaviors of these metal oxide NPs.

The simultaneous environmental exposure of soils to different NPs is likely to occur for example after sewage sludge application. The impact of a combination of NPs has been

investigated using Ag, Cu, and Si NPs in an arctic soil (Kumar et al. 2011). In this study, a significant decrease of microbial biomass was observed after 6 months of incubation for a mixture concentration of 660 mg kg^{-1} soil (220 mg kg^{-1} of each NP).

Impact of carbon nanoparticles (fullerene and CNTs)

Contrasting effects on microbial biomass were reported for carbon nanoparticles. Johansen et al. (2008) found no effect, whereas decreases were observed by other authors but only for concentrations exceeding 250 mg kg^{-1} of CNTs (Chung et al. 2011; Jin et al. 2013; Rodrigues et al. 2013). These negative effects seemed to be more severe on fungal biomass compared to bacterial biomass (Rodrigues et al. 2013). These effects observed only with high concentrations are consistent with the results previously presented on the microbial activities, suggesting a limited toxicity of carbon NPs toward soil microbial communities.

Impact of nanoscale zero valent iron

The effects of nZVI have been investigated on the abundance of microbial functional groups to detect potential modifications that could be detrimental for ecosystem functioning. The presence of 34 and 10 g nZVI kg^{-1} soil triggered decrease in denitrifying bacteria abundance (Fajardo et al. 2012) and in chloroaromatic mineralizing microorganisms (Tilston et al. 2013), respectively. These results illustrate that a high concentration of nZVI could affect soil nitrogen cycle and the biodegradative potential of a microbial functional group. Pawlett et al. (2013) reported that nZVI addition reduced microbial biomass but only when soil was amended with 5 % straw. These results suggest that the impact of NPs may be dependent of the organic matter content of soil, which has been found to enhance NPs mobility in porous media (Ben-Moshe et al. 2010; Wang et al. 2012; Thio et al. 2011). Organic matter may be an important factor favoring NPs bioavailability for soil microorganisms. However, the number of studies available on the mobility and toxicity of NPs in natural soils with contrasted textures and organic matter contents is too limited to confirm this assumption (Fang et al. 2009; Pawlett et al. 2013; Cornelis et al. 2013; Peyrot et al. 2014; Simonin et al. 2014).

Impact of nanoparticles on microbial diversity

The measurement of the microbial biomass is a black box approach in which the genetic and functional diversity among the microbial community are not considered. Soil hosts an immense diversity of microorganisms (individual taxa commonly described as "operational taxonomic units"; OTUs) of bacteria, fungi, and archaea. Microbial diversity encompasses

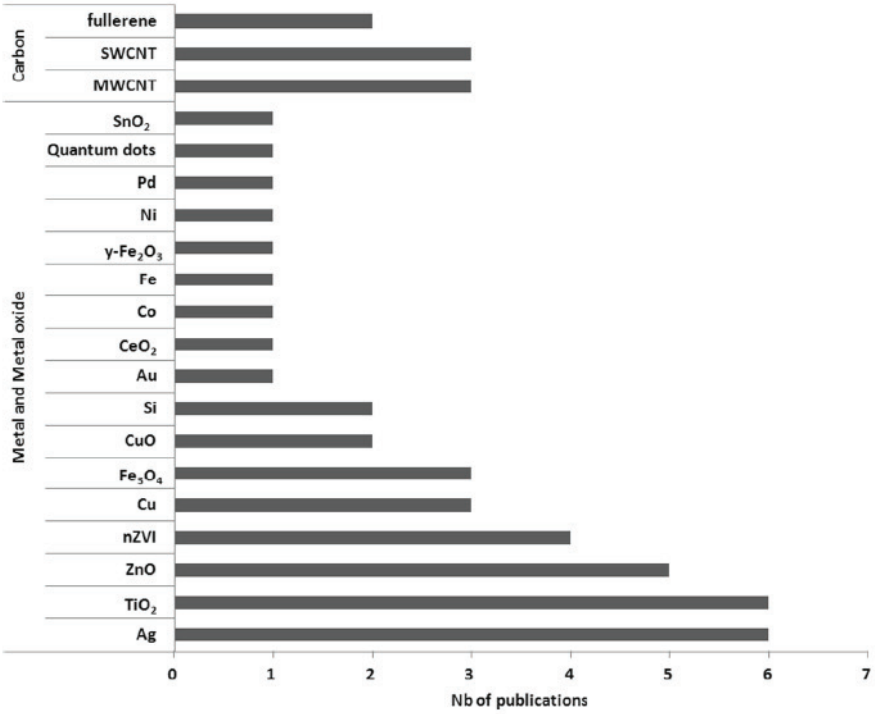


Fig. 1 Number of publications studying the impact of metal/metal oxide or carbon nanoparticles on soil microbial communities. Thirty-one publications were available in July 2014

genetic variability within taxa (species), and the number (richness) and the relative abundance (evenness) of taxa and functional groups (guilds) in communities (Torsvik and Øvreås 2002). That is why, investigating the impact of NPs on microbial diversity is crucial to provide information on how and why soil ecosystem functioning is affected. A panel of techniques, such as PLFA profiles, PCR-DGGE, T-RFLP analysis, or next generation sequencing, has been used to evaluate the impact of NPs on microbial diversity.

Impact of metal and metal oxide nanoparticles

Ag-NPs altered bacterial community structure, after short-term exposure of sewage sludge containing 0.14 mg kg^{-1} of Ag-NP (Colman et al. 2013). Kumar et al. (2011) also observed that Ag-NPs could modify bacterial community in an arctic soil but with a higher concentration (660 mg kg^{-1}). A field study also reported that zero valent Cu and ZnO-NPs had no effect on PLFA profiles and on microbial community composition as determined by pyrosequencing (Collins et al. 2012). Changes in soil bacterial community composition due to the presence of Fe_3O_4-NPs were observed after a short incubation (24 or 48 h) as well as after 15 and 30 days (Ben-Moshe et al. 2013; He et al. 2011). Cloning–sequencing of DGGE bands indicated a stimulation of specific groups of *Actinobacteria*, *Duganella*, *Streptomycetaceae*, or *Nocardioides* (He et al. 2011). These groups facilitate the decomposition of organic matter, which could explain the concomitant soil invertase and urease increases measured during this experiment. Different concentrations of TiO_2 and ZnO-NPs decreased soil bacterial diversity after 60 days of incubation (Ge et al. 2011; Ge et al. 2012). The pyrosequencing data indicated that some of the declining taxa are known to be associated to nitrogen fixation (*Rhizobiales*, *Bradyrhizobiaceae*, and *Bradyrhizobium*) and methane oxidation (*Methylobacteriaceae*), while some positively impacted taxa are known to be associated to the decomposition of recalcitrant organic pollutants (*Sphingomonadaceae*) and biopolymers including protein (*Streptomycetaceae* and *Streptomyces*). The role of these taxa for soil functioning suggest potential consequences on ecosystem-scale processes. Nogueira et al. (2012) assessed the effect of five inorganic nanomaterials (TiO_2, $TiSiO_4$, CdSe/ZnS quantum dots, gold nanorods, and Fe/Co magnetic fluid) on soil bacterial

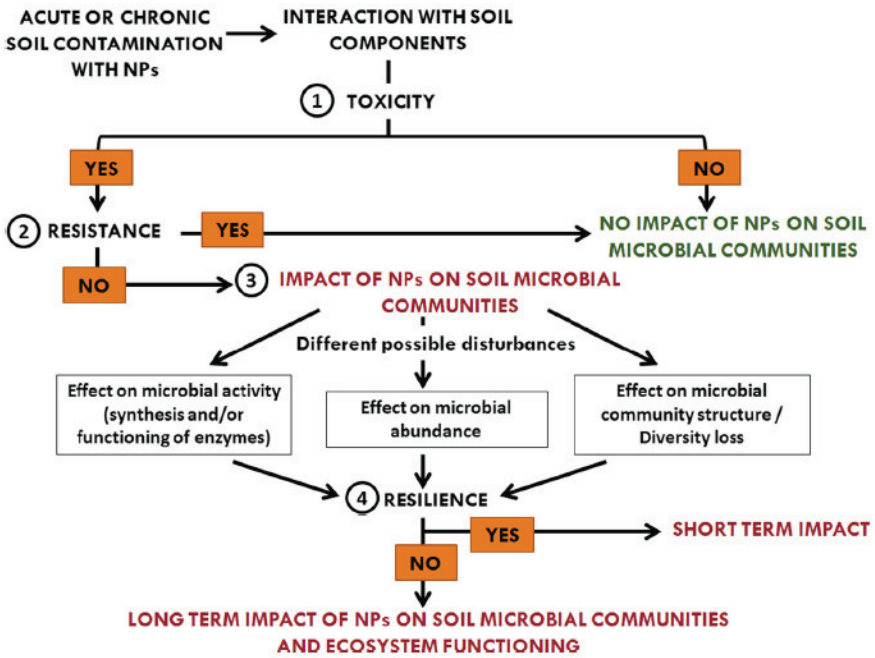


Fig. 2 The response of soil microbial communities to NPs contamination. *1* Soil properties (clay, organic matter content, pH, ionic strength…) can modulate the toxicity of NPs for microorganisms. *2* If the NPs can be toxic, the impact on microbial community will depend on the ability of the community to resist to this perturbation. *3* If the indigenous microorganisms are not resistant to NPs contamination, soil microbial communities could be affected through different nonexclusive mechanisms: (i) effects of NPs on microbial activities due to an alteration of the synthesis and/or functioning of enzymes, (ii) a modification of microbial abundance caused by a mortality of sensitive population in presence of NPs, and (iii) a modification of microbial community structure and/or a diversity loss. *4* Soil microbial communities may become tolerant to NPs contamination and may be resilient to this disturbance. If resilience is observed, the impact of NPs will be on relatively short-term duration. If no or limited resilience is observed, NPs could have long-lasting effects on soil microbial communities with cascading effects on ecosystem functioning, especially biogeochemical cycles, soil fertility, and climate regulation

Table 2 Review of the effects of carbon nanoparticles on soil microbial communities

Carbon nanoparticles (fullerene and CNTs)					
Reference	NPs	NPs concentrations	Soil	Experiment	Major results
Tong et al. (2007)	FullereneC_{60}	1 mg kg^{-1} soil in aqueous suspension or 1000 mg kg^{-1} soil in granular form	Silty-clay-loam (USA)	Microcosms- up to 180 days	No effect of fullerene on soil respiration, enzymatic activities PLFA profiles or DGGE profiles.
Johansen et al. (2008)	FullereneC_{60}	0, 5, 25, 50 mg kg^{-1} soil	Sandy-clay-loam (Denmark)	Microcosms - 0, 7, 14 days	No effect of fullerene on soil respiration, microbial biomass, and on the enumeration of protozoans. Decrease of bacterial enumeration only immediately after contamination. Limited effects of fullerene on DGGE profiles(15-30 % dissimilarity between treatments) for both Bacteria and Protozoans.
Chung et al. (2011)	MWCNT	50, 500, 5000 mg kg^{-1} soil	2 sandy soils (Korea)	Microcosms – 0, 1, 4, 11 days	In both soil types, most enzyme activities decreased at 500 mg kg^{-1} soil, and all enzymatic activities as well as microbial biomass C and N were significantly lowered at 5000 mg kg^{-1} soil.
Khodakovskaya et al. (2013)	MWCNT	50 or 200 µg mL^{-1} added once a week	Redi-earth Plug and Seedling Mix (Sun Gro horticulture, Inc.)	Experimental pot seeded with tomato – 9 weeks	No decrease of bacterial diversity was observed but a significant modification of the bacterial composition was found (DGGE analysis). The relative abundances ofBacteroidetes and Firmicutes increased, whereas Proteobacteria andVerrucomicorbia decreased with increasing concentration of MWCNT.
Shrestha et al. (2013)	MWCNT	10, 100, 1000 mg kg^{-1} soil	Sandy loam (USA)	Jars - 28 and 90 days	No effects of MWNTs on soil respiration, enzymatic activities, and microbial community composition. Decreased abundance of some bacterial genera like Derxia, Holophaga, Opitutus and Waddlia at the highest treatment while bacterial genera that are considered potential degraders of recalcitrant contam-inants (such as polycyclic aromatic hydrocarbons) like Rhodococcus, Cellulomonas, Nocardioides and Pseudomonas increased (Pyrosequencing data).
Rodrigues et al. (2013)	SWCNT	0, 250, 500 mg kg^{-1} soil	Sandy loam (USA)	Microcosms - 3, 7, 14 days incubation	SWCNT reduced the bacterial abundance and modified carbon substrate utilization after 3 days but not after 7 and 14 days. Decrease of fungal abundance and modification of the fungal community structure (T-RFLP analysis)at every date of soil exposure to SWCNT.
Jin et al. (2013)	SWCNT	0, 30,100,300,600 and 1000 mg SWCNT kg^{-1} soil	Sandy loam (Korea)	Microcosms - 25 days	Decreases of most enzymatic activities and microbial biomass for the higher concentrations (300,600 and 1000 mg SWCNT kg^{-1} soil).
Jin et al. (2014)	SWCNT	0, 30,100,300,600 and 1000 mg SWCNT kg^{-1} soil.	Sandy loam (Korea)	Microcosms - 25 days	High concentrations of SWCNTs significantly altered soil microbial community composition. Gram-positive and Gram-negative bacterial and fungal biomass decreased with higher SWCNT concentrations.

Table 3 Review of the effects of nanoscale zero valent iron nanoparticles on soil microbial communities

Nanoscale zero valent iron (nZVI)					
Reference	NPs	NPs concentrations	Soil	Experiment	Major results
Cullen et al. (2011)	nZVI dispersed with polyacrylic acid	10 g kg^{-1} soil	Silt loam soil (UK)	Microcosms -0, 1, 3, 7 and 14 days	nZVI apparently inhibited microbial ammonia-oxidation and stimulated dehydrogenase activity but had minimalinfluence on hydrolase activity. The authors found that nZVI reactivity (redox potential) caused confounding effect on the activity measurements and thus no evidence for negative effects of nZVI on ammonia oxidation and deshydrogenase activities was found.
Fajardo et al. (2012)	nZVI	34 g Fe kg^{-1} soil	Sandy clay loam	Microcosms -72 h	nZVI treatment increasedArchaea, α-proteobacteria and Gram+C abundances but decreasedβ andγ-protéobacteria abundances measured by FISH. Abundance of denitrifying bacteria (gene *narG* and *nirS*) was also reduced in presence of nZVI but *gyrA* gene copy number increased (qPCR). The expression of these 3 genes was not modified in the nZVI treatment.
Pawlett et al. (2013)	nZVI stabilized with sodium CMC	270 mg Fe kg^{-1} soil	3 soils: sandy, loam and clay amended with 3 concentrations of straw (0, 5 et 10 % organic matter) (UK)	Microcosms– 4 months	Microbial biomass was decreased by nZVI only in the soils amended with 5 % straw. Microbial community structure (PLFA profiles) was modified in the 3 soils: sandy (at 0, 5 and 10 % OM), loam (0 and 10 % OM) and clay soil (5 % OM). Negative effects on Gram negative bacteria and arbuscular mycorrhizalfungi. Decrease of Multiple Substrate Induced respiration (MSIR) was only observed in the clay soil. No effect was found on basal metabolic rate.
Tilston et al. (2013)	polyacrylic acid (PAA)-coated nZVI	10 g kg^{-1} soil	Silt loam soil (UK)	Microcosms - 0, 1, 4, 7 and 28 days	nZVI was associated with altered soil bacterialcomposition (DGGE profiles) and reduced chloroaromatic biodegradation activityas demonstrated by mineralization assay using 2,4-D as a modelchloroaromatic.

community structure using DGGE. After 30 days of soil exposure, TiO_2 and gold nanorods induced the highest changes in the structural diversity of bacterial community. The limited effect of $TiSiO_4$, CdSe/ZnS quantum dots, and Fe/Co magnetic fluid NPs on DGGE profiles was attributed to their zeta potential values reflecting an unstable state. Hence, once added to the soil, they may have interacted with soil components, becoming unavailable to exert toxic effects.

Metal or metal oxide NPs may be responsible for a biodiversity loss and a modification of soil microbial community composition. Although many research focused on the effect of these NPs on bacterial communities, more work is still required to assess the impact of NPs on fungal and archaeal communities.

Impact of carbon nanoparticles (fullerene and CNTs)

Fullerene NPs had no effect on microbial diversity (Tong et al. 2007) or induced only slight modification of *Eubacteria* and *Kinetoplastida* (Protozoans) community structure on DGGE profiles (20 to 30 % of dissimilarity, Johansen et al. 2008). Pyrosequencing data indicated that MWCNT (10 g kg^{-1} soil) induced an enrichment of potential degraders of recalcitrant contaminants (PAH) *Rhodococcus*, *Cellulomonas*, *Norcardioles*, and *Pseudomonas*, while some bacterial genera like *Derxia*, *Holophaga*, *Opitutus*, and *Waddlia* were decreased (Shrestha et al. 2013). Using a comparative metagenomic analysis of bacterial communities, Khodakovskaya et al. (2013) found that the diversity and richness of bacterial communities were not affected by MWCNTs, while a significant modification of the bacterial composition was observed. SWCNTs induced a modification of microbial community composition resulting in a decrease of Gram-positive and -negative bacterial biomass and fungal biomass as well (Jin et al. 2014). Rodrigues et al. (2013) reported also a modification of the fungal community structure after 14 days of soil exposure to SWCNT (250 and 500 mg kg^{-1}). Consistent with activity and abundance measurements, carbon NPs can alter soil microbial community structure but only in presence of high concentrations (>250 mg kg^{-1}).

Impact of nanoscale zero valent iron

The impact of nZVI on microbial diversity was investigated using FISH, DGGE, and PLFA analysis. Fajardo et al. (2012) did not report a broad bactericidal effect of nZVI but observed significant shifts in the structure and phylogenetic composition of the soil microbial community after 72 h of incubation. The FISH assays provided evidence that nZVI exerts a selective pressure on the microbial community, promoting the dominance of some microbial groups (*Archaea*, α-*Proteobacteria*, and low G+C Gram-positive bacteria) or the decrease of other ones (β- and γ-*Proteobacteria* and subclasses). DGGE profiles also indicated a significant modification of bacterial community composition after 28 days in the presence of 10 g kg^{-1} of nZVI (Tilston et al. 2013). Pawlett et al. (2013) observed that nZVI caused a modification of PLFA profiles in all soil texture tested, but that these effects were modulated by the organic matter content of the soil. These studies suggest that nZVI could induce a significant modification of soil microbial community structure, affecting bacteria, archaea, and fungi populations on the short term (<4 months).

Concluding remarks

NPs toxicity toward microorganisms has been demonstrated in numerous in vitro studies (e.g., Simon-Deckers et al. 2009; Jiang et al. 2009); yet, the assessment of NPs environmental impact is still in its early stages. This synthesis on the effects of NPs on soil microbial communities supports different conclusions.

It has been demonstrated using different methodologies, different indicators, and natural soils, which NPs could have an impact on microbial activities, abundances, and diversity. However, this synthesis highlights that the toxic effects on microbial community are highly dependent on both the NPs considered and the soil properties. In fact, inorganic NPs (metal, metal oxide NPs) may have a greater toxic potential than organic NPs (fullerenes and CNTs) to soil microorganisms. An exception could be the iron oxide magnetic NPs, which exhibit limited negative effects on microbial communities even when high concentrations were applied. Alarmingly, the use of nZVI could have detrimental effects on biodegradative functions of microorganisms in a context of soil remediation. Further research is needed to determine the real efficiency of nZVI treatments in soil and their potential consequences on soil functioning using activity and functional gene measurements.

The soil properties seem to play a key role for the bioavailability of NPs, especially the clay and organic matter content. We strongly encourage to take more into consideration the physicochemical characteristics of the soil used in the experiments (texture, organic matter content, pH…) and to compare the ecotoxicity of NPs in a range of different soils. The identification of soil parameters controlling the bioavailability of NPs is fundamental for a better environmental risk assessment (Cornelis et al. 2014).

It should be noted that the effects of numerous NPs have not been investigated yet or only in a single study (Al_2O_3, CeO_2, quantum dots, SiO_2, SnO_2…) (Fig. 1), whereas a significant amount of these NPs is susceptible to be released to soils (Keller et al. 2013). Some NPs have been more studied than others (Ag, TiO_2, ZnO) (Fig. 1), and paradoxically, these are not necessarily the most produced and used NPs (Keller

et al. 2013; Sun et al. 2014). The overall number of publications on each class of NP remains still limited to date (≤6) (Fig. 1), and thus, it is still difficult to generalize the results. More research is needed, especially through experiments using more environmentally realistic concentrations of NPs based on the predicted concentrations in modelization studies and using more realistic exposure conditions. All the literature analyzed in this review assessed the acute toxicity of NPs corresponding to a sudden disturbance due to a unique application of NPs and the monitoring of the response of microorganisms over time. These experiments assessed the short-term sensitivity of microbial communities, but to date, no data are available neither on the long-term effect and the chronic toxicity of NPs nor on the ability of microorganisms to be resilient to NP disturbance over time.

Although this review emphasizes that microbial ecotoxicology is a valuable approach for risk assessment of NPs on soil ecosystem functioning, the use of microorganisms as indicators of the NPs impact in soil is still in its infancy. At least three reasons might be evoked to explain this fact; first, this is a recent concern steadily increasing since 2011 (Table 1), second this is a complex multidisciplinary subject requiring both biological and physicochemical approaches, and third, it suffers from current technical limitation that hampered our knowledge on NPs behavior in soil. The following section details some crucial subjects that must be investigated to improve our understanding of the environmental impact of these materials using microbial ecotoxicology.

Future research needs

Characterization and fate of NPs in soil

It is unadvisable to conduct ecotoxicological studies without analytical data of the used pollutant. To date, NPs have been extensively characterized in spiking suspensions usually prepared in ultrapure water, in aquaticenvironments, but not directly in the soil, because of the current technical limitations to detect NPs in complex media (Tourinho et al. 2012; Cornelis et al. 2014). To understand bioavailability and toxicity of NPs, more effort are needed to determine the fate of NPs, i.e., the speciation, the mobility, the homoaggregation and heteroaggregation processes, and all the physicochemical and biological transformations that NPs can undergo in the soil. These issues have been addressed in several reviews (see Lowry et al. 2012; Pan and Xing 2012; Cornelis et al. 2014). The crucial point for the assessment of NP toxicity to soil microorganisms relies on the accurate measurement of the bioavailable fraction of NPs in soil. As discussed by Cornelis et al. (2014), current techniques to estimate the bioavailable fraction of NPs in complex environments are unsatisfactory and need further developments.

Evaluation of a realistic exposure of soil to NPs

Currently, most of the data on the effect of NPs on soil microorganisms were obtained under unrealistic exposure conditions, using NP concentrations 50- to 10,000-fold higher than modeled concentrations in soils (Gottschalk et al. 2009; Sun et al. 2014). Few studies used NPs concentrations ≤1 mg kg^{-1} soil and most of them concerned Ag-NP (Table 1). More research using realistic concentrations is needed especially since it has been demonstrated that these low concentrations can still have detrimental effects for soil microbial communities (Hänsch and Emmerling 2010; Colman et al. 2013; Simonin et al. 2014).

Microcosm experiments enable assessment of the impact of NPs under controlled conditions (temperature, soil humidity…) on short-term monitoring. Mesocosms are particularly effective tools to assess the impact of pollutants on ecosystem functioning under realistic conditions and long-term monitoring. Few studies performed experiments in mesocosms and/or under field conditions to simulate more realistic exposures of soils to NPs. Moreover, the addition of NP in suspension prepared in ultrapure water used in most experiments is not a realistic mode of soil exposure. A likely route by which NPs enter soil is as aged NPs through sewage sludge applications for field fertilization. Further research in these directions will bring useful information to set regulations for the manufacture, use, and disposal of NPs.

Resilience of soil microbial communities to NPs disturbance

This review highlights that soil microorganisms are sensitive to acute contamination of NPs. However, because of the lack of long-term experiments, the resiliency of soil microbial communities to such disturbance and the return to the predisturbance activity levels after sustainable changes in microbial abundance and diversity along time remains still unknown. Allison and Martiny (2008) demonstrated that the composition of most microbial groups is sensitive and not immediately resilient to disturbance, regardless taxonomic breadth of the group or the type of disturbance. This also appears to be true for NP contamination, because in several studies, a greater effect was observed over time, but in period generally not exceeding 2 months (e.g., Ge et al. 2011; Colman et al. 2013). The response of soil microorganisms to NPs needs to be monitored over a long-term periods to evaluate whether ecosystem functioning is permanently disturbed or not (Fig. 2).

From a more realistic point of view, chronic contamination of NPs should be simulated. To date, no study using chronic addition of NPs is available. It is likely that NPs are added to soil chronically through multiple applications of sewage sludge over time. Microbial communities are likely to respond differently to chronic and acute exposures. Acute exposures

may lead to more resistant microbial communities to NP perturbation if sensitive communities are replaced by resistant communities. On the contrary, chronic exposures may have more detrimental effects if microorganisms are not resilient to such repeated contaminations. Studying if resilience of soil microbial community occurs after acute or chronic contaminations with NPs is crucial to evaluate possible consequences on soil functioning over time (Fig. 2).

Effect of mixture toxicity on soil microbial communities

There is a growing interest in the impact of mixture toxicity in soil since most pollutants share some identical routes of entry in soil (wastewater irrigation, sewage sludge application…) and occur simultaneously in polluted sites. With the increasing use of manufactured NPs, it is necessary that NPs are considered in mixture toxicity studies along with heavy metals, hydrocarbons, or antibiotics. Moreover, it is even more crucial because some NPs are ableto mobilize some pollutants. In a context of remediation, this ability may be beneficial, but when not controlled, NPs may enhance the bioavailability of other pollutants and caused detrimental effects.

Assessing the impact of NPs in soil exhibiting different physicochemical properties

Soil is a complex matrix and extrapolation of results from one contaminated soil to another is difficult because of the great variability in soil composition and structure (Ranjard et al. 1997). However, most experiments were conducted on a single model soil (Table 1), which does not permit a comparison of soil sensitivity to NPs. Soil properties, such as pH, texture, or organic matter content, influence microbial community composition (Fierer and Jackson 2006) and bioavailability of pollutants (Giller et al. 1998). Thus, the knowledge of the soil physicochemical properties influencing NPs bioavailability (Fig. 2) will greatly enhance our understanding of NPs impact on soil functioning. In addition, we have little information on the spatialization and mobility of NPs in soil (Fang et al. 2009; Vittori Antisari et al. 2013), but like heavy metals, hot spots of pollutants may occur (Ranjard and Richaume 2001). Microscale approaches are needed to determine if NPs have a heterogeneous impact in soil. Moreover, the use of microbial indicators may be useful to have better insights on NPs spatialization to circumvent the current lack of techniques to characterize NPs in situ (Tables 2 and 3).

Assessing fungal and archaeal response to NPs

More attention is classically given to soil bacterial communities, despite the crucial role of fungal communities in energy flow and nutrient transfer in terrestrial ecosystems. New insights regarding the broad distribution and abundance of archaea in soils and oceans imply that they also contribute to global energy cycles, especially nitrogen cycle where ammonia-oxidizing archaea play a key role in nitrification (Schleper et al. 2005; Robertson et al. 2005; Zhang et al. 2010). What is known on NPs toxicity from in vitro studies using bacterial strains may not be extrapolated to archaeal and fungal communities. Thus, it is important to consider the response of archaeal and fungal communities to NPs contamination, in order to have a better assessment of NPs ecotoxicity in soils.

Acknowledgments Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region–ARC Environnement. This work was funded by a grant from the French National Program Microbien EC2CO-CNRS. The authors are grateful to Dr Sylvie Nazaret for her critical reading of the manuscript.

References

Allison SD, Martiny JB (2008) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A 105:11512–11519

Ben-Moshe T, Dror I, Berkowitz B (2010) Transport of metal oxide nanoparticles in saturated porous media. Chemosphere 81:387–393

Ben-Moshe T, Frenk S, Dror I, Minz D, Berkowitz B (2013) Effects of metal oxide nanoparticles on soil properties. Chemosphere 90:640–646

Brookes PC (1995) The use of microbial parameters in monitoring soil pollution by heavy metals. Biol Fertil Soils 19:269–279

Chung H, Son Y, Yoon TK, Kim S, Kim W (2011) The effect of multi-walled carbon nanotubes on soil microbial activity. Ecotoxicol Environ Saf 74:569–575

Colman BP, Arnaout CL, Anciaux S, Gunsch CK, Hochella MF Jr, Kim B, Lowry GV, McGill BM, Reinsch BC, Richardson CJ, Unrine JM, Wright JP, Yin L, Bernhardt ES (2013) Low concentrations of silver nanoparticles in sewage sludge cause adverse ecosystem responses under realistic field scenario. PLoS ONE 8:e57189

Collins D, Luxton T, Kumar N, Shah S, Walker VK, Shah V (2012) Assessing the impact of copper and zinc oxide nanoparticles on soil: A field study. PLoS ONE 7:e42663

Cornelis G, Pang L, Doolette C, Kirby JK, McLaughlin MJ (2013) Transport of silver nanoparticles in saturated columns of natural soils. Sci Total Environ 463:120–130

Cornelis G, Hund-Rinke K, Kuhlbusch T, Van den Brink N, Nickel C (2014) Fate and bioavailability of engineered nanoparticles in soils: a review. Crit Rev Environ Sci Technol 44:2720–2764

Cullen LG, Tilston EL, Mitchell GR, Collins CD, Shaw LJ (2011) Assessing the impact of nano-and micro-scale zerovalent iron particles on soil microbial activities: particle reactivity interferes with assay conditions and interpretation of genuine microbial effects. Chemosphere 82:1675–1682

Dinesh R, Anandaraj M, Srinivasan V, Hamza S (2012) Engineered nanoparticles in the soil and their potential implications to microbial activity. Geoderma 173:19–27

Du WC, Sun YY, Ji R, Zhu JG, Wu JC, Guo HY (2011) TiO_2 and ZnO nanoparticles negatively affect wheat growth and soil enzyme activities in agricultural soil. J Environ Monit 13:822–828

Fabrega J, Fawcett SR, Renshaw JC, Lead JR (2009) Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter. Environ Sci Technol 43:7285–7290

Fajardo C, Ortíz LT, Rodríguez-Membibre ML, Nande M, Lobo MC, Martin M (2012) Assessing the impact of zero-valent iron (ZVI) nanotechnology on soil microbial structure and functionality: A molecular approach. Chemosphere 86:802–808

Fang J, Shan XQ, Wen B, Lin JM, Owens G (2009) Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. Environ Pollut 157:1101–1109

Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci U S A 103:626–631

Ge YG, Schimel JP, Holden PA (2011) Evidence for negative effects of TiO_2 and ZnO nanoparticles on soil bacterial communities. Environ Sci Technol 45:1659–1664

Ge YG, Schimel JP, Holden PA (2012) Identification of soil bacteria susceptible to TiO_2 and ZnO nanoparticles. Appl Environ Microbiol 78:6749–6758

Ge YG, Priester JH, Van De Werfhorst LC, Schimel JP, Holden PA (2013) Potential Mechanisms and Environmental Controls of TiO_2 Nanoparticle Effects on Soil Bacterial Communities. Environ Sci Technol 47:14411–14417

Giller KE, Witter E, Mcgrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol Biochem 30:1389–1414

Gottschalk F, Sonderer T, Scholz RW, Nowack B (2009) Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Environ Sci Technol 43: 9216–9222

Griffiths BS, Philippot L (2013) Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev 37:112–129

Hänsch M, Emmerling C (2010) Effects of silver nanoparticles on the microbiota and enzyme activity in soil. J Plant Nutr Soil Sci 173: 554–558

He S, Feng Y, Ren H, Zhang Y, Gu N, Lin X (2011) The impact of iron oxide magnetic nanoparticles on the soil bacterial community. J Soils Sediments 11:1408–1417

Holden PA, Schimel JP, Godwin HA (2014) Five reasons to use bacteria when assessing manufactured nanomaterial environmental hazards and fates. Curr Opin Biotechnol 27:73–78

Jiang W, Mashayekhi H, Xing B (2009) Bacterial toxicity comparison between nano-and micro-scaled oxide particles. Environ Pollut 157: 1619–1625

Jin L, Son Y, Yoon TK, Kang YJ, Kim W, Chung H (2013) High concentrations of single-walled carbon nanotubes lower soil enzyme activity and microbial biomass. Ecotoxicol Environ Saf 88:9–15

Jin L, Son Y, DeForest JL, Kang YJ, Kim W, Chung H (2014) Single-walled carbon nanotubes alter soil microbial community composition. Sci Total Environ 446:533–538

Johansen A, Pedersen AL, Jensen KA, Karlson U, Hansen BM, Scott-Fordsmand JJ, Winding A (2008) Effects of C60 fullerene nanoparticles on soil bacteria and protozoans. Environ Toxicol Chem 27: 1895–1903

Ju-Nam Y, Lead JR (2008) Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications. Sci Total Environ 400:396–414

Kandeler E, Kampichler C, Horak O (1996) Influence of heavy metals on the functional diversity of soil microbial communities. Biol Fertil Soils 23:299–306

Keller AA, McFerran S, Lazareva A, Suh S (2013) Global life cycle releases of engineered nanomaterials. J Nanopart Res 15:1692

Khodakovskaya MV, Kim BS, Kim JN, Alimohammadi M, Dervishi E, Mustafa T, Cernigla CE (2013) Carbon nanotubes as plant growth regulators: effects on tomato growth, reproductive system, and soil microbial community. Small 9:115–123

Klaine SJ, Alvarez PJ, Batley GE, Fernandes TF, Handy RD, Lyon DY et al (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem 27:1825–1851

Kumar N, Shah V, Walker VK (2011) Perturbation of an arctic soil microbial community by metal nanoparticles. J Hazard Mater 190: 816–822

Kumar N, Shah V, Walker VK (2012) Influence of a nanoparticle mixture on an arctic soil community. Environ Toxicol Chem 31:131–135

Lowry GV, Gregory KB, Apte SC, Lead JR (2012) Transformations of nanomaterials in the environment. Environ Sci Technol 46: 6893–6899

Naja G, Apiratikul R, Pavasant P, Volesky B, Hawari J (2009) Dynamic and equilibrium studies of the RDX removal from soil using CMC-coated zerovalent iron nanoparticles. Environ Pollut 157:2405–2412

Neal AL (2008) What can be inferred from bacterium–nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? Ecotoxicology 17:362–371

Nogueira V, Lopes I, Rocha-Santos T, Santos AL, Rasteiro GM, Antunes F, Gonçalves F, Soares AMVM, Cunha A, Almeida A, Gomes NNCM, Pereira R (2012) Impact of organic and inorganic nanomaterials in the soil microbial community structure. Sci Total Environ 424:344–350

Pan B, Xing B (2012) Applications and implications of manufactured nanoparticles in soils: a review. Eur J Soil Sci 63:437–456

Pawlett M, Ritz K, Dorey RA, Rocks S, Ramsden J, Harris JA (2013) The impact of zero-valent iron nanoparticles upon soil microbial communities is context dependent. Environ Sci Pollut Res 20: 1041–1049

Peyrot C, Wilkinson KJ, Desrosiers M, Sauvé S (2014) Effects of silver nanoparticles on soil enzyme activities with and without added organic matter. Environ Toxicol Chem 33:115–125

Ranjard L, Richaume A, Jocteur-Monrozier L, Nazaret S (1997) Response of soil bacteria to Hg (II) in relation to soil characteristics and cell location. FEMS Microbiol Ecol 24:321–331

Ranjard L, Richaume A (2001) Quantitative and qualitative microscale distribution of bacteria in soil. Res Microbiol 152:707–716

Robertson CE, Harris JK, Spear JR, Pace NR (2005) Phylogenetic diversity and ecology of environmental Archaea. Curr Opin Microbiol 8: 638–642

Rodrigues DF, Jaisi DP, Elimelech M (2013) Toxicity of functionalized single-walled carbon nanotubes on soil microbial communities: implications for nutrient cycling in soil. Environ Sci Technol 47:625–633

Rousk J, Ackermann K, Curling SF, Jones DL (2012) Comparative toxicity of nanoparticulate CuO and ZnO to soil bacterial communities. PLoS ONE 7:e34197

Satapanajaru T, Anurakpongsatorn P, Pengthamkeerati P, Boparai H (2008) Remediation of atrazine-contaminated soil and water by nano zerovalent iron. Water Air Soil Pollut 192:349–359

Schleper C, Jurgens G, Jonuscheit M (2005) Genomic studies of uncultivated archaea. Nat Rev Microbiol 3:479–488

Schloter M, Dilly O, Munch JC (2003) Indicators for evaluating soil quality. Agric Ecosyst Environ 98:255–262

Schimel JP, Schaeffer SM (2012) Microbial control over carbon cycling in soil. Front Microbiol. doi:10.3389/fmicb.2012.00348

Shah V, Belozerova I (2009) Influence of metal nanoparticles on the soil microbial community and germination of lettuce seeds. Water Air Soil Pollut 197:143–148

Shah V, Collins D, Walker VK, Shah S (2014) The impact of engineered cobalt, iron, nickel and silver nanoparticles on soil bacterial diversity under field conditions. Environ Res Lett 9:024001

Shin YJ, Kwak JI, An YJ (2012) Evidence for the inhibitory effects of silver nanoparticles on the activities of soil exoenzymes. Chemosphere 88:524–529

Shrestha B, Acosta-Martinez V, Cox SB, Green MJ, Li S, Cañas-Carrell JE (2013) An evaluation of the impact of multiwalled carbon nanotubes on soil microbial community structure and functioning. J Hazard Mater 261:188–197

Simon-Deckers A, Loo S, Mayne-L'hermite M, Herlin-Boime N, Menguy N, Reynaud C et al (2009) Size-, composition-and shape-

dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria. Environ Sci Technol 43: 8423–8429

Simonin M, Guyonnet JP, Martins JMF, Ginot M, Richaume A (2014) Influence of soil properties on the toxicity of TiO_2 nanoparticles on carbon mineralization and bacterial abundance. J Hazard Mater. doi: 10.1016/j.jhazmat.2014.10.004

Sun TY, Gottschalk F, Hungerbühler K, Nowack B (2014) Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. Environ Pollut 185:69–76

Thio BJR, Zhou D, Keller AA (2011) Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles. J Hazard Mater 189:556–563

Tilston EL, Collins CD, Mitchell GR, Princivalle J, Shaw LJ (2013) Nanoscale zerovalent iron alters soil bacterial community structure and inhibits chloroaromatic biodegradation potential in Aroclor 1242-contaminated soil. Environ Pollut 173:38–46

Tong Z, Bischoff M, Nies L, Applegate B, Turco RF (2007) Impact of fullerene (C60) on a soil microbial community. Environ Sci Technol 41:2985–2991

Torsvik V, Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240–245

Tourinho PS, Van Gestel CA, Lofts S, Svendsen C, Soares AM, Loureiro S (2012) Metal-based nanoparticles in soil: Fate, behavior, and effects on soil invertebrates. Environ Toxicol Chem 31:1679–1692

Vittori Antisari L, Carbone S, Gatti A, Vianello G, Nannipieri P (2013) Toxicity of metal oxide (CeO_2, Fe_3O_4, SnO_2) engineered nanoparticles on soil microbial biomass and their distribution in soil. Soil Biol Biochem 60:87–94

Wang Y, Gao B, Morales VL, Tian Y, Wu L, Gao J, Yang L (2012) Transport of titanium dioxide nanoparticles in saturated porous media under various solution chemistry conditions. J Nanopart Res 14:1–9

Xia T, Kovochich M, Liong M, Mädler L, Gilbert B et al (2008) Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 2:2121–2134

Zhang L-M, Offre PR, He J-Z, Verhamme DT, Nicol GW, Prosser JI (2010) Autotrophic ammonia oxidation by soil thaumarchaea. Proc Natl Acad Sci U S A 107:17240–17245

5. Questions de la thèse

Cette synthèse bibliographique a permis de mettre en évidence les avancées récentes et les verrous qui restent à lever pour mieux comprendre le devenir et l'impact des nanomatériaux dans les sols. Afin d'évaluer les risques liés à deux NPs d'oxydes métalliques (TiO_2 et CuO), nous nous sommes intéressés en parallèle (i) à leur devenir, en considérant leur transport et leurs caractéristiques physico-chimiques dans les sols, et (ii) à leur écotoxicité, en évaluant leur impact sur l'activité, l'abondance et la diversité des communautés microbiennes du sol. Plus particulièrement, les questions auxquelles nous avons souhaité répondre dans cette thèse sont les suivantes :

- Quelle est l'influence des propriétés du sol, notamment la texture et la teneur en matière organique, sur la mobilité des NPs d'oxydes métalliques ?
- Quelle est l'influence des propriétés du sol, en particulier de la texture et de la teneur en matière organique, sur la toxicité des NPs d'oxydes métalliques sur les communautés microbiennes du sol?
- Quel est l'effet de ces NPs sur les groupes fonctionnels microbiens nitrifiants et dénitrifiants impliqués dans le cycle de l'azote, dont les niveaux de redondance fonctionnelle sont différents?
- Les faibles concentrations ont-elles des effets négatifs sur le fonctionnement microbiologique du sol ?
- Existe-t-il une relation dose-réponse associée à l'impact des NPs sur les communautés microbiennes du sol ?
- Le transport et la toxicité des NPs dans les sols sont-ils modifiés lors d'apports chroniques et aigus ?

6. Méthodologie générale

Afin de répondre à ces questions, des choix méthodologiques ont été nécessaires. Ils sont présentés et justifiés ci-dessous.

a. Les nanoparticules d'oxydes métalliques

Nous avons travaillé avec des NPs pures de TiO_2 et de CuO (Figure 13). Comme dit précédemment, le TiO_2 est le nanomatériau le plus représenté dans les sols en raison de son utilisation dans de nombreux produits de grande consommation (cosmétiques, peintures, aliments, médicaments…). Contrairement aux TiO_2-NPs, les CuO-NPs sont moins fréquemment utilisées et sont donc vraisemblablement présentes dans les sols en plus faibles concentrations. Cependant, elles présentent des propriétés antimicrobiennes bien connues (produits anti-fouling, antibactériens…) et présentent l'un des plus forts potentiels toxiques *in vitro* comparés aux autres NPs (Baek and An 2010 ; Li et al. 2012). C'est pourquoi, il nous a paru intéressant de les prendre en compte dans cette thèse.

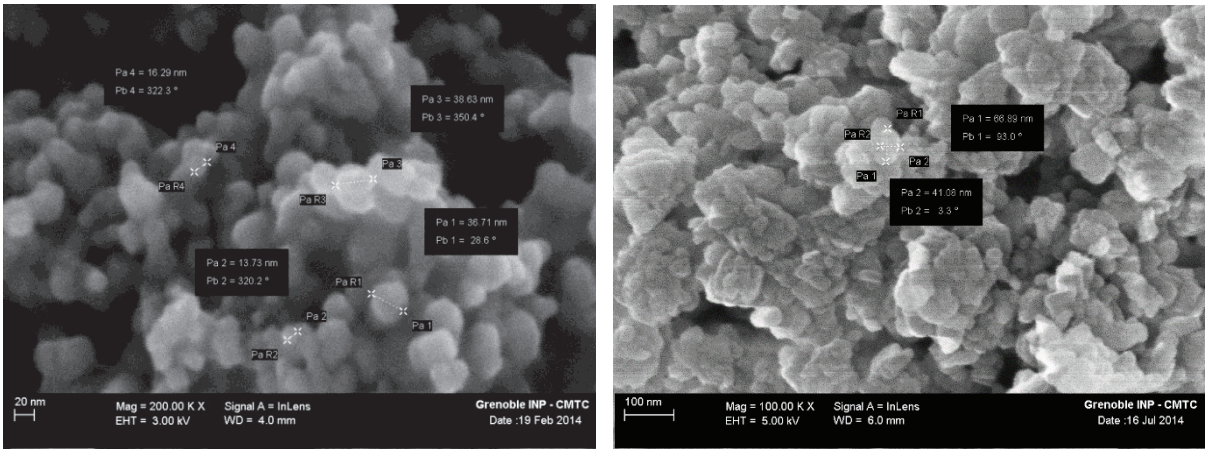


Figure 13: Images de microscopie électronique à balayage (SEM-FEG, Zeiss, CMTC, G-INP) des nanoparticules de TiO_2 (à gauche) et de CuO (à droite) utilisées lors de la thèse.

b. Les sols

Nous avons choisi de travailler avec 6 sols agricoles présentant des propriétés physico-chimiques contrastées en termes de texture (teneur en argile, limon et sable) et/ou de teneur en MO. Trois classes texturales ont été étudiées (sablo-limoneuse, limoneuse et limono-argileuse). Pour chacune d'elles, deux sols ayant des teneurs en MO différentes (i.e.

faible ou forte) ont été prélevés sur un même site. Cette sélection de sols, nous a permis de tester l'influence de 3 textures contrastées sur le transport ou la toxicité des NPs et pour chaque texture, d'apprécier l'influence de la teneur en MO. Les historiques d'utilisation de ces sols sur plusieurs dizaines d'années sont connus car ils font partie de stations expérimentales suivies au laboratoire ou font l'objet d'un suivi dans le cadre du réseau de mesures de la qualité des sols (RMQS – GIS Sol).

c. Les dispositifs expérimentaux

L'étude de la mobilité des NPs de TiO_2 et du CuO a été effectuée en utilisant une approche classique en colonnes de sols saturés en eau dans lesquelles sont injectées en entrée les NPs selon une fonction connue (Figure 14). La fonction de sortie ou courbe de percée est déterminée en analysant les effluents de la colonne, ce qui permet de déterminer la proportion de NPs transportées dans la colonne de sol étudiée en comparaison avec la quantité injectée dans le sol.

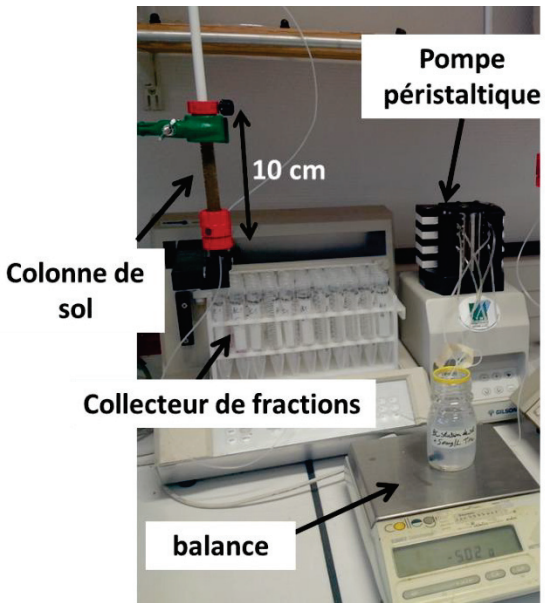


Figure 14: Dispositif expérimental utilisé pour étudier le transport des nanoparticules dans des colonnes de sol (Photo : Marie Simonin). Les colonnes sont en verre et mesurent 1 cm de diamètre et 10 cm de long (Ge-Healthcare Pharmacia Biotech Inc.).

L'étude de la toxicité des NPs de TiO_2 et CuO a été réalisée, dans la plupart des expérimentations, en microcosmes de sols qui ont été contaminés de façon homogène (Figure 15). Cette approche a permis de travailler en conditions contrôlées de température, d'humidité et d'obscurité sur des durées d'exposition allant jusqu'à 90 jours.

Figure 15: Incubation de microcosmes (flacon plasma 150 mL) contenant 50 g sol sec dans une étuve à 28°C (Photo : Marie Simonin).

d. Les indicateurs microbiens

La toxicité des NPs de TiO_2 et du CuO a été déterminée en étudiant l'activité, l'abondance et la diversité des communautés microbiennes qui sont des indicateurs pertinents et complémentaires pour évaluer la qualité d'un sol et sa réponse à une perturbation. Notre but était de déterminer si les NPs pouvaient altérer le fonctionnement biologique du sol (i.e. les activités microbiennes dans notre cas) et, le cas échéant, de comprendre pourquoi le processus mesuré était affecté, en s'intéressant aux effets sur l'abondance, la structure des communautés et la diversité des groupes fonctionnels impliqués (Article 1 : Figure 2).

Nous nous sommes donc intéressés à des processus microbiens globaux et restreints (Schimel *et al.*, 2005 : broad and narrow processes). Nous avons retenu, comme indicateur global, la respiration du sol (ou minéralisation du carbone), qui est réalisée par une grande diversité de microorganismes aérobies et hétérotrophes. Elle est mesurée comme un processus unique par dégagement de CO_2 mais est en réalité la somme d'une multitude de processus impliqués dans la dégradation de la matière organique du sol, qui représente le flux majeur de carbone de ces écosystèmes. Afin de comprendre les effets observés sur la respiration du sol (mesurée par la technique SIR, substrate-induced respiration), nous avons étudié en parallèle les effets sur l'abondance et la diversité bactérienne totale du sol. Les effets sur l'abondance et la diversité des communautés fongiques n'ont pas été pris en compte dans cette thèse.

Des processus microbiens restreints impliqués dans le cycle de l'azote ont été considérés : la nitrification (oxydation de NH_4^+ en NO_3^-) et la dénitrification (réduction séquentielle du NO_3^- en N_2). Ces processus sont réalisés par des groupes de microorganismes qui sont

responsables seulement d'une part du cycle total de l'élément impliqué. Ces groupes fonctionnels sont généralement peu diversifiés et représentent une faible proportion de la communauté microbienne totale (Schimel and Schaeffer, 2012). Par exemple, la nitrification repose sur 2 étapes qui sont la nitritation et la nitratation (Figure 16). Chacune de ces étapes est réalisée par des groupes fonctionnels microbiens distincts. La nitritation est réalisée par les archées et les bactéries oxydatrices de l'ammonium (AOA et AOB, respectivement), alors que la nitratation est effectuée dans les sols par des bactéries oxydatrices du nitrite (NOB) appartenant aux genres *Nitrobacter* et *Nitrospira*.

Notre démarche a consisté à évaluer les effets des NPs sur les activités potentielles de nitrification et de dénitrification, ainsi que sur l'abondance et la diversité des groupes fonctionnels impliqués dans ces deux processus en ciblant des gènes de fonction.

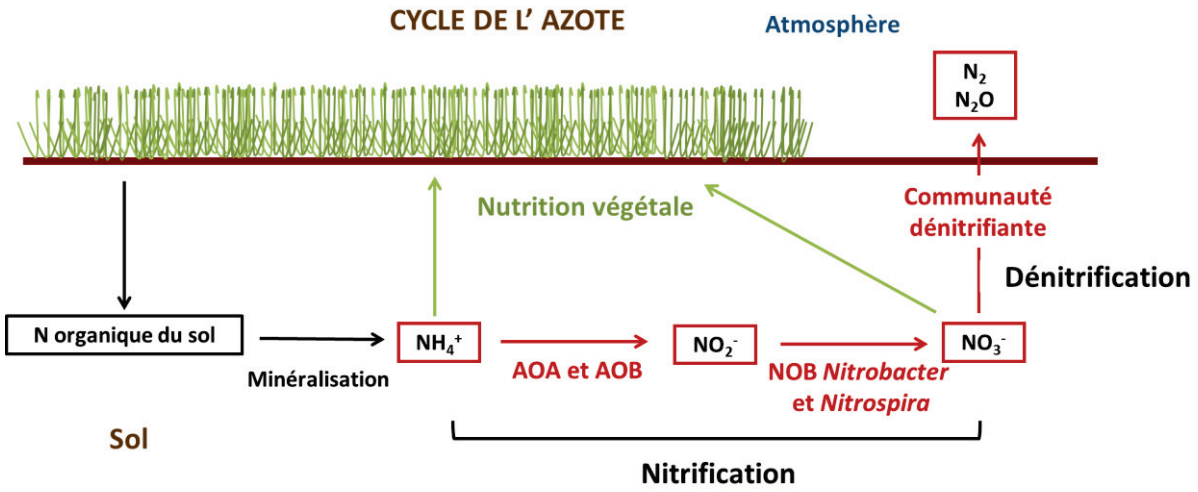


Figure 16: Schéma simplifié du cycle de l'azote dans les sols. Les groupes fonctionnels microbiens impliqués dans la nitrification et la dénitrification sont indiqués en rouge.

L'étude des activités microbiennes (respiration, nitrification et dénitrification) nous a permis d'évaluer l'impact des NPs sur des processus clés des cycles du carbone et de l'azote, essentiels pour la fertilité des sols. Cela nous a également permis de déterminer si l'impact des NPs était différent sur des activités microbiennes reposant sur des groupes fonctionnels possédant des degrés de redondance fonctionnelle différents (le nombre d'espèces capables de réaliser la même fonction). En se basant sur l' « insurance hypothesis » proposée par Loreau *et al.*, (2002), on s'attend à ce qu'une plus grande diversité au sein d'un groupe fonctionnel confère une meilleure résistance du processus associé à une perturbation environnementale. Cette hypothèse se base sur l'idée intuitive que la probabilité de trouver

des espèces capables de s'adapter lors de perturbations et contribuant à la fonction est plus grande dans un groupe ayant une forte diversité. Dans notre cas, on pourrait donc s'attendre à ce que le processus le plus sensible aux NPs soit la nitrification (faible redondance) et le plus résistant, la respiration du sol (forte redondance).

Chapitre 2 : Influence des propriétés du sol sur le transport et la toxicité des nanoparticules d'oxydes métalliques

1. Introduction

Il existe une grande diversité de sols en termes de texture, structure, pH ou de teneur en MO par exemple. Ces propriétés physico-chimiques variées vont influencer le devenir des NPs dans les sols. Ces propriétés influencent également la composition et la physiologie des communautés microbiennes du sol. De ce fait, on peut faire l'hypothèse que le devenir et l'impact des NPs va différer d'un sol à un autre en fonction de sa composition physico-chimique et de la communauté microbienne qu'il héberge.

Toutefois, à l'heure actuelle peu d'études ont étudié le devenir des NPs dans différents types de sols naturels et la majorité des études d'écotoxicologie microbienne a été réalisée sur un seul sol modèle (Article 1). Dans le cadre de l'évaluation des risques associés à ces polluants émergents dans les écosystèmes terrestres, il serait nécessaire d'identifier des paramètres physico-chimiques clés du sol qui influencent les propriétés des NPs et donc leur mobilité et leur toxicité.

Nous nous proposons donc dans ce chapitre d'évaluer l'influence de la texture et de la teneur en MO du sol sur la mobilité (Article 2) et la toxicité des NPs (Article 3). Pour cela, des approches expérimentales en colonnes et en microcosmes ont été mises en place en étudiant 6 sols agricoles aux propriétés physico-chimiques contrastées.

2. Influence des propriétés du sol sur le transport du TiO_2 et CuO en colonnes de sol

a. Article 2 : Présentation générale de l'étude et synthèse des principaux résultats

La majorité des études disponibles sur le transport des NPs a été réalisée dans des milieux poreux modèles (sable, billes). Ces études ont montré l'importance de considérer l'influence de facteurs abiotiques, tels que la MO, la force ionique, l'argile, pour comprendre la mobilité de ces polluants. Toutefois, ces milieux poreux modèles ne représentent pas les conditions complexes retrouvées dans des sols naturels, en termes de variété des types de surfaces minérales et organiques, de chimie de la solution du sol, ou de granulométrie.

C'est pourquoi, dans cette étude nous avons étudié le transport des TiO_2 et CuO-NPs dans des colonnes de 6 sols naturels présentant des textures et teneurs en MO différentes. Les concentrations en titane et cuivre retrouvées dans les effluents des colonnes ont été mesurées après digestion acide et quantification par ICP-OES, afin de déterminer la part mobile des NPs et la part retenue dans le sol.

Nous avons mis en évidence que les CuO et TiO_2-NPs avaient une mobilité faible à très faible dans les 6 sols étudiés, avec 86 à 98% des NPs retenues dans les colonnes de sol. Ni la texture ni la teneur en matière organique n'ont d'effet notable sur le transport du TiO_2. En revanche, pour les CuO-NPs dans les sols sablo-limoneux et limono-argileux le transport était contrasté entre les sols avec faible ou forte teneur en MO. Dans le sol sablo-limoneux, la mobilité du CuO était plus importante dans le sol présentant une forte teneur en MO, alors que l'inverse était observé dans le sol limono-argileux. Ces différences de mobilité ne semblent pas liées à des différences d'agrégation ou de charge de surface du CuO-NPs mais plutôt à une forte concentration en carbone organique dissout dans la solution du sol qui pourrait favoriser le transport des NPs.

Nos résultats indiquent que la mobilité des NPs d'oxydes métalliques, évaluée sur une distance de 10 cm, est très limitée et donc que le transfert de ces polluants vers les couches profondes du sol ou les eaux souterraines serait négligeable. Nous n'avons pas pu mettre en évidence d'effet en lien avec la porosité des différents sols car dans les sols sablo-limoneux et limono-argileux, les taux de rétention des NPs étaient similaires.

b. «Influence of clay and organic matter content on the transport of TiO_2 and CuO nanoparticles in saturated soil columns»

L'article 2 intitulé « Influence of clay and organic matter content on the transport of TiO_2 and CuO nanoparticles in saturated soil columns » sera soumis dans le journal *Environmental Pollution*.

Influence of clay and organic matter content on the transport of TiO_2 and CuO nanoparticles in saturated soil columns

Marie SIMONIN[1,2,3,4], Jean M. F. MARTINS[1*], Gaëlle UZU[1], Alix DUMAY[1], Aline NAVEL[1] and Agnès RICHAUME[,2,3,4]

[1] UJF-Grenoble/CNRS-INSU/G-INP/IRD, LTHE UMR 5564, Grenoble F-38041, France

[2] Université de Lyon, Lyon, France

[3] Université Claude Bernard Lyon 1, Villeurbanne, France

[4] CNRS, UMR 5557, Ecologie Microbienne, Université Lyon 1, Villeurbanne, France

[*]Correspondence to: jean.martins@ujf-grenoble.fr

ABSTRACT

The transport of metal-oxide nanoparticles (CuO and TiO_2) in 6 natural soils was studied in saturated columns. In particular, we investigated the influence of clay and organic matter (OM) content on their transport. For this purpose, breakthrough curves of CuO and TiO_2 were measured in soils belonging to 3 different textural classes (sandy-loam, loam and silty-clay) and for each of them, two contrasted OM content.

CuO was mobile in all soils but the retention was generally high (86% minimum). No clear influence of soil texture could be observed on CuO transport, but in the sandy-loam and silty-clay soils, it was affected by the OM content. In all soil solutions, CuO dissolution was negligible (0.14 to 0.49 %) suggesting that the Cu concentration measured in the effluent could mainly be attributed to CuO nanoparticules and not to its dissolved form. The TiO_2-NPs mobility in all soil columns was found to be very low with a mass recovery value of 1.5 – 5.5% and was always lower than CuO, which may be related to the higher homoaggregation of TiO_2 in soil solutions. Due to its high retention in soil, no clear influence of soil texture or OM content could be observed on the TiO_2 transport.

Our result show that CuO and TiO_2 nanoparticles exhibit a low mobility in a range of natural soils and that dissolved organic carbon is certainly a key driver of CuO mobility in soil.

Key-words: Soils, Nanomaterials, Transfer, Dissolved organic carbon, CDE modelling

INTRODUCTION

Metal oxide engineered nanoparticles (NPs) are becoming integral elements of a myriad of consumer and industrial products. Copper oxide (CuO) NPs are used extensively in catalysts, superconductors or in paints for their biocidal properties (Zhu *et al.*, 2004; Ren *et al.*, 2009; Keller *et al.*, 2013). Titanium dioxide (TiO_2) NPs are commonly integrated in personal care products, paints or food packaging (Keller *et al.,* 2014). As a result, soils are exposed to these components through various exposure routes, which may lead to potential risks for humans and ecosystems health. Their release into soils can occur during industrial production, landfills, agricultural amendments of sewage sludge, abrasion of materials and accidental spill (Nowack *et al.*, 2012). Therefore, it appears urgent to improve our current understanding of the fate and transport of NPs in soils in order to better assess the risks for groundwater and agricultural soils.

Transport of NPs has been largely investigated in well-defined simple porous media, such as glass beads or quartz sand, but few investigations have addressed the transport of metallic NPs in natural soils (Fang *et al.*, 2009, 2011; Cornelis *et al.*, 2013). Soils are porous systems consisting in complex structured assemblies of mineral and organic particles combined with liquid and gaseous phases. Several soils components such as clay and organic matter (OM) are known to influence the mobility of colloids because of their important reactive surfaces, which can interact with particles and modify their physicochemical behavior (Pan and Xing, 2012). Several studies have demonstrated the influence of these factors on NPs aggregation, surface charge and stability (Fang *et al.*, 2009; Thio *et al.*, 2011; Zhou *et al.*, 2012). Dissolved OM is known to favor NPs dispersion and thus NPs mobility in simple porous media (Ben-Moshe *et al.*, 2010; Thio *et al.*, 2011), while clay particles can destabilize positively and negatively charged NPs and thus decrease their mobility (Zhou *et al.*, 2012). Moreover, these processes are greatly influenced by the chemistry of the soil solution, such as pH, ionic strength and electrolytes (Wang *et al.*, 2012; Cornelis *et al.*, 2014; Vitorge *et al.*, 2014). Fang *et al.,* (2011) demonstrated that TiO_2-NPs could act as a metal carrier and facilitate for instance the mobilization of Cu in soil. These results raise some concern about the potential environmental risk associated with enhanced migration rates of co-contaminants and the increased bioavailability of some toxic metals in presence of NPs. Due to the interactions between these multiple environmental factors in natural soils, it is difficult to predict the

fate of NPs in soil from investigations conducted in simple porous media. In particular it remains highly difficult to hierarchize the dominant factors controlling the transfer of manufactured NPs in natural soils, since most of these factors are closely linked. Studies evaluating the simultaneous effects of several soil factors on NPs mobility are clearly needed to elucidate the key soil properties controlling NPs mobility.

The objectives of this study were to determine the influence of soil texture and OM content on the transport in natural soils of two relevant manufactured NPs, CuO and TiO_2. We conducted saturated column experiments with 6 agricultural soils of contrasted physicochemical properties: 3 textures: sandy-loam, loam and silty-clay, and 2 different OM contents for each soil texture. The NPs breakthrough curves were fitted with the Convection Dispersion Equation (CDE) in order to identify the dominant processes involved in these NPs transfer in soil and quantify the corresponding parameters.

MATERIALS AND METHODS

Soils

This study was conducted with soils belonging to three different textural classes: a sandy-loam, a loam and a silty-clay. Soils were collected from the upper 20 cm layer of 3 different agricultural sites located in the Rhône-Alpes and Burgundy regions of France (see Article 3: Simonin *et al.,* 2015 for further details). At each site, the sampling was performed in 2 plots with low and high OM contents distant of less than 500 m. Therefore, the experiments were performed with 6 test soils: with 3 different textures and 2 different OM contents for each texture. After collection, visible rocks, roots and plant litter were manually removed. The soils were sieved (2 mm) and homogenized before storage at 4°C. Soils were previously characterized (Simonin *et al.*, 2015). Some selected soil physicochemical properties are presented in Table 1.

Soil solutions of the 6 soils were prepared following Simonin *et al.,* (2015) and used as background solutions during the column experiments. Briefly, soil solutions were prepared by shaking 10 g of soil dispersed in 50 mL of ultrapure water (18MΩ) during 30 minutes at 180 rpm and 20°C in a refrigerated incubator shaker (New Brunswick - Eppendorf, Hamburg,

Germany). The solutions were then centrifuged for 20 min at 8000 g, 20°C (Centrifuge 5804R, Eppendorf, Hamburg, Germany) to eliminate particles larger than 20 nm according to the Stokes' law. The supernatants were collected and stored at 4°C before use in column experiments.

Table 1 Main characteristics of the six studied soils

	Soils										Soil solutions		
	% sand	% loam	% clay	% OM	% WHC	pH	Ionic strength (mM)	CEC ($cmol^+ kg^{-1}$)	Cu ($mg\ kg^{-1}$)	Ti ($g\ kg^{-1}$)	pH	Ionic strength (mM)	DOC ($mg\ L^{-1}$)
Sandy-loam - Low OM	68.4	14.7	16.9	2.09	20	7.0	0.98	11.5	20.1	2.4	6.7	1.0	11.6
Sandy-loam - High OM	65.6	16.1	18.3	4.46	20	6.9	1.59	15.4	21.9	2.4	6.4	2.0	18.6
Loam - Low OM	37.5	42.7	19.8	2.23	30	6.4	0.60	8.79	13.2	2.7	6.2	1.2	8.2
Loam - High OM	40.3	40.8	18.9	6.77	30	6.3	1.31	15.3	10.4	2.4	5.4	2.4	10.5
Silty-clay - Low OM	8.2	49.8	42.0	4.72	47	6.9	0.51	17.4	23.2	5.3	6.3	1.5	25.2
Silty-clay - High OM	10.1	50.8	39.1	7.87	51	7.7	1.37	20.1	26.2	4.5	7.1	1.6	8.9

Nanoparticles

TiO_2-NPs were provided by Sigma Aldrich (St Louis, USA) as a mixture of anatase (80%) and rutile (20%) crystal structure with at least 99.5% purity. According to the manufacturer information, TiO_2-NPs presented a specific surface area of 35-65 $m^2\ g^{-1}$ and a mean particle size of 21 nm in powder as measured by Transmission Electron Microscopy. CuO-NPs were purchased from Sigma Aldrich (St Louis, USA) with a nominal size < 50 nm and a specific surface area of 23 $m^2\ g^{-1}$, according to the manufacturer information. Both NPs were characterized for intrinsic primary particle size using a ZEISS Ultra 55 scanning electron microscopy – field emission gun (SEM-FEG). In average, TiO_2-NPs measured 28.7 ± 7.1 nm and CuO-NPs measured 57.0 ± 18 nm.

The apparent hydrodynamic diameter and zeta potential of the NPs were characterized using Dynamic Light Scattering (DLS) in the soil solutions used as background solutions. The measurements were performed in triplicate in the spiking suspensions of NPs (soil solution + NPs at 50 $mg\ L^{-1}$), after dispersion through ultrasonication for 5 min to ensure suspensions homogeneity.

Column experiments

The transport experiments were performed in small glass columns (C10/10, GE Healthcare) of 1 cm in diameter and 10 cm long, homogeneously packed with 8 cm of wet soil. Flow adaptors (AC 10, GE Healthcare) were adjusted on the top of the columns to ensure a constant soil height during experiments. At the beginning of the experiments, soil columns were saturated and leached with 100 mL of soil solution. The effluents were collected using a fraction collector to check for the turbidity of the outflow by measuring the absorbance at 600 nm (Spectrometer Biowave II, Biochrom WPA). The absorbance was very low for all soils (< 0.02), suggesting that the amount of soil colloids in the effluents was negligible. Bromide (Br) ion was used as water tracer to assess the hydrodynamic properties of the soil columns before NPs injection. One pore volume of Br tracer (KBr 1 g L^{-1} prepared in soil solution) was injected in each soil column and followed by 2 pore volumes injection of soil solution. The concentrations of Br in the soil column effluents were measured by Ion chromatography (Metrohm 732/733 separation center) with a detection limit (DL) of 4 µg L^{-1}.

The spiking suspensions of NPs (TiO_2 or CuO) were prepared at 50 mg L^{-1} (C0) in soil solution before the beginning of the experiment and were injected in the columns at a flow rate of 0.1 mL min^{-1} during 5 pore volumes. After NPs spiking, the columns were continuously leached with the soil solution for at least 15 pore volumes. The column effluents were sampled in 15 mL centrifuge tubes every 10 minutes (~1 mL). Titanium or copper concentrations were measured in the spiking suspensions (C0) and in the effluents (C) to construct breakthrough curves of NPs presented in a dimensionless form: C/C0 as a function of V/V0, the dimensionless eluted volume, V, normalized by the total water volume of the column, V0. The NPs analysis were performed using microwave assisted (Novawave, SCP Science) strong acid extraction (for titanium: hydrofluoric acid + nitric acid and for copper: *aqua regia*). Elements concentrations were determined using inductively coupled plasma optical emission spectrophotometer (ICP-OES; Varian 700-ES, Varian Inc. Scientific Instruments, Palo Alto, USA). With this methodology, titanium and copper concentrations measured in a certified soil reference material were in good agreement with certified values (data not shown). The detection limit was 50 µg L^{-1} for both Cu and Ti measurements. Control columns without NPs injection were performed for each soil to determine the

background concentration of titanium and copper in the effluents. The physicochemical properties of the soils columns are provided in Table 2.

To evaluate the occurrence of CuO dissolution in spiking suspensions during each column experiment, a dissolution test was performed. The 6 spiking suspensions (one for each soil) were prepared and sampled (5 mL) after 6 hours corresponding to the maximum duration of the NPs column transport experiments. Dissolved Cu concentration in samples were determined by ICP-OES (Varian 700-ES) after centrifugation for 20 min at 6000 g in a 5 kDa ultrafiltration device (Vivaspin tube, Sartorius) in triplicate. Soil solutions without CuO-NPs were used as controls. The dissolution of TiO_2-NPs in spiking suspensions was not assessed because these NPs are not soluble in water (Chen and Mao, 2007; Duester *et al.*, 2011).

Table 2 Main characteristics of the soil columns

	Pore volume (mL)	Bulk density (g cm^{-3})	Effective Porosity	Darcy velocity (cm h^{-1})	Average soil collector diameter (µm)
Sandy-loam - Low OM	4.3	1.27	0.51	0.38	122.9
Sandy-loam - High OM	4.8	1.08	0.59	0.38	118.3
Loam - Low OM	5.1	0.83	0.68	0.25	74.5
Loam - High OM	5.4	0.86	0.67	0.25	78.9
Silty-clay - Low OM	5.9	1.01	0.61	0.16	24.9
Silty-clay - High OM	6.7	0.94	0.64	0.15	28.4

Transport parameters estimation

The CXTFIT code implemented in the STANMOD software (USDA) (Toride *et al.*, 1995) was used to model tracer and NPs transport experiments. The pore water velocity (V, cm h^{-1}) and dispersion (D, cm^2 s^{-1}) in the NPs transport simulations were determined from the Br tracer breakthrough curves. The analytic solution of the Convection Dispersion Equation (CDE) with a first-order loss term presented in Eq.1 was fitted to the Br and NPs breakthrough curves using the Levenberg Maquardt algorithm (e.g. Martins and Mermoud, 1998; Martins *et al.*, 2013; Vitorge *et al.*, 2013; Lakshmanan *et al.*, 2015).

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial z^2} - V\frac{\partial C}{\partial z} - \mu C \qquad \text{Eq.1}$$

where C is the concentration, t is time, z is distance from the inlet, V is average pore water velocity, D is dispersion coefficient and μ is the decay coefficient, related to NPs irreversible retention on solid collectors. For Br experiments V, R (retardation factor, dimensionless) and D were calculated. For NPs experiments the parameters V, R, D and μ were calculated for the fitting of the breakthrough curves.

Statistical analysis

The results of NPs hydrodynamic diameter, zeta potential and dissolution in soil solutions are presented as means (± standard errors) of 3 replicates. The influence of soil texture and OM content on NPs hydrodynamic diameter and zeta potential was investigated with a two-way Anova using the R software.

RESULTS AND DISCUSSION

Physicochemical properties of nanoparticles in soil solutions

CuO and TiO_2-NPs were characterized in the 6 soil solutions used as spiking suspensions during the transport experiments (Figure 1). The TiO_2-NPs hydrodynamic diameter values ranged from 73.9 to 128.1 nm and a significant effect of OM content and of the interaction of OM content and soil texture was observed on this parameter (Figure 1A, Table 3). These significant effects were related to the higher aggregation of TiO_2-NPs in the silty-clay soil (High OM). This higher aggregation could be attributed to the low DOC content and the high concentration of Ca in relation with the alkaline pH of this soil solution (Table 1, Figure 1A) (Simonin *et al.*, 2015; Badawy *et al.*, 2010; French *et al.*, 2009). The CuO-NPs hydrodynamic diameter values ranged from 53.3 to 84.1 nm, meaning that almost no aggregation of CuO-NPs occurred in soil solution because their diameter in powder was 57 ± 18 nm in average. Thus, CuO-NPs size was lower than TiO_2-NPs in all soil solutions, except in the loam soil (High OM) (Figure 1B). No significant influence of soil texture or OM content on CuO-NPs aggregation was observed (Table 3).

Both TiO_2 and CuO-NPs were negatively charged in all soil solutions (Figure 1C and 1D) and these zeta potential values were significantly affected by soil texture and OM content (Table

3). TiO_2-NPs zeta potential ranged from -15.5 to -23.9 mV and these zeta potential values in soil solutions with low OM content were always more negatively charged than in high OM content soil. CuO-NPs zeta potential ranged from –13.8 nm to -20.8 mV and conversely to TiO_2-NPs, the CuO-NPs in soil solution with low OM content were always less negatively charged than in high OM content soil, probably in relation with the high affinity of copper for OM (Jacobson *et al.*, 2007; Morel *et al.,* 2014; Navel and Martins, 2014).

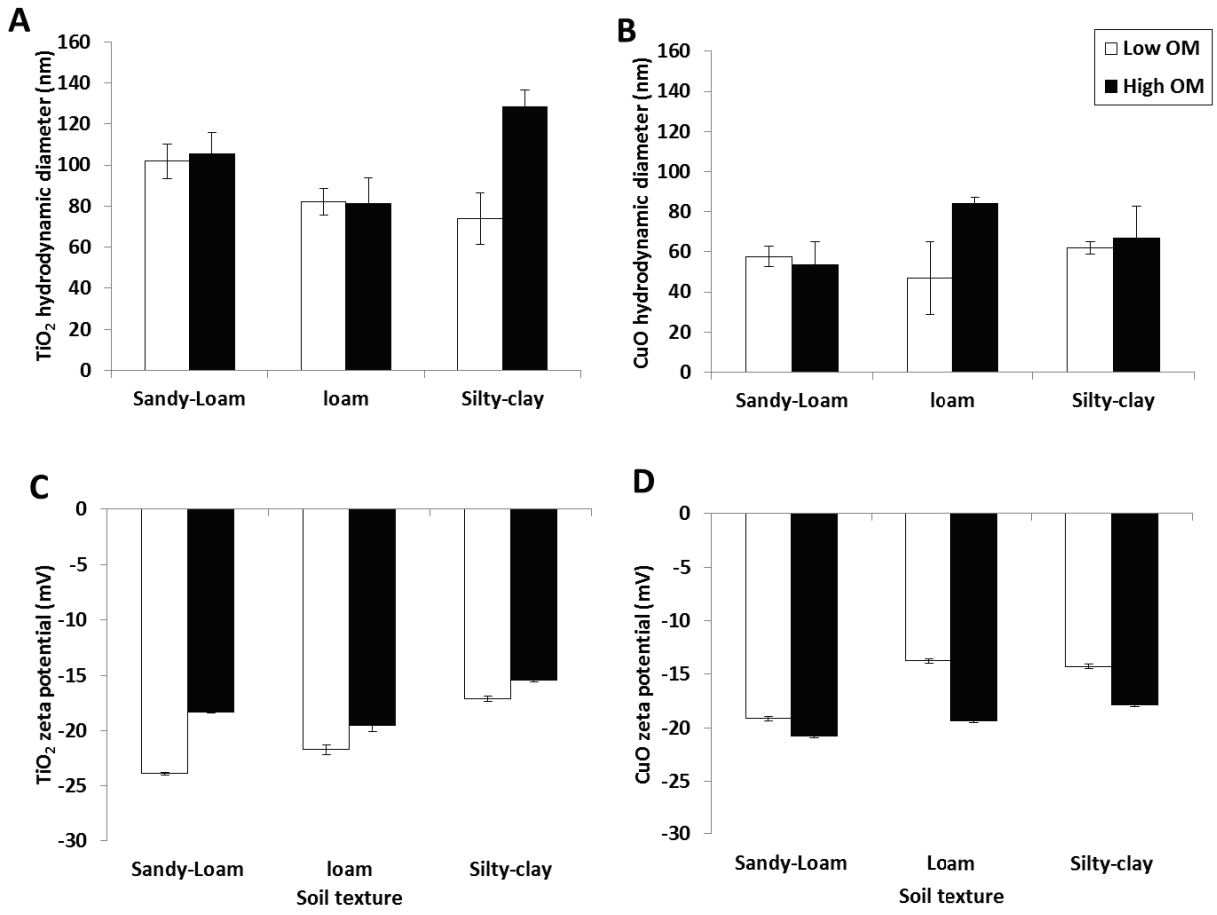


Figure 1 TiO_2 and CuO-NPs hydrodynamic diameter (A and B, respectively) and zeta potential (C and D, respectively) measured in background soil solutions prepared at 50 mg NPs L^{-1} before transport experiments.

Table 3 *P*-values associated to the effect of soil texture, OM content and the interaction of texture and OM on the size (i.e. hydrodynamic diameter) and zeta potential of TiO_2 and CuO-NPs.

	TiO_2		CuO	
	Size	**Zeta potential**	**Size**	**Zeta potential**
Texture	0.12	**< 0.001**	0.64	**< 0.001**
OM content	**0.04**	**< 0.001**	0.19	**< 0.001**
Texture x OM	**0.04**	**< 0.001**	0.20	**< 0.001**

Hydrodynamic properties of the 6 soils

The hydrodynamic properties of the 6 soils were determined by fitting the Br breakthrough curves with the CXTFIT code (Figure 2). The corresponding transport parameters are presented in Table 4 along with those fitted for the CuO-NPs.

All tracer breakthrough curves presented mass recoveries close to 100%, as expected with a water tracer. Retardation (R) factors were all close to 1 except that calculated in the Silty-clay low OM, in which R was 0.82, indicating that part of the water in soil was not seen by the tracer, in relation with the high clay content of this soil. The dispersion values (D) fitted to the breakthrough curves are quite low (below 10^{-7} cm^2 s^{-1}) and very similar for all soils. These parameter values were used to fit the breakthough curves of the NPs.

Transport of CuO-NPs in soil columns

The dissolution of CuO-NPs was assessed in the spiking suspensions. CuO dissolution was negligible in all soil solutions, as it ranged between 0.14 and 0.49 % of the NPs applied mass after 6 hours, representing the duration of NPs injection in soil columns. These results show that Cu concentrations measured in the effluents can be attributed to CuO-NPs.

The observed and calculated breakthrough curves of CuO-NPs are presented in Figure 2. The corresponding parameters are presented in Table 4. The breakthrough curves of CuO-NPs show that these NPs were strongly retained in the 6 studied soils with quite low mass recoveries ranging between 5.2 and 14% (Table 4).

No clear influence of soil texture on CuO-NPs transport could be observed. In particular, CuO-NPs transport was not specifically higher in the sandy-loam soils than in the loam and silty-clay soils that have higher clay contents, as it could be expected from previous works (Fang *et al.*, 2009). However, in the sandy-loam and silty-clay soils, the OM content affected

the transport of CuO-NPs (Figure 2), while in the loam soils the breakthroughs were very similar in both low and high OM soils (Figure 2). Interestingly in the sandy-loam soil, the mobility increased of 62% in the high OM soil compared to the low OM soil, whereas in the silty-clay soil, CuO-NPs mobility decreased of 44% in the high OM soil compared to the low OM soil (Table 4, Figure 2).

In the sandy-loam (high OM) and the silty-clay (low OM) soils, where CuO-NPs transport was enhanced, the DOC concentration in soil solution was higher than in the other soils (Table 1). Several soil factors may be involved in the lower retention of CuO-NPs in these two soils, however DOC concentration appears as a main driver of CuO-NPs mobility, as already demontrated in model porous media (Ben-Moshe *et al.*, 2010). The difference of zeta potential of CuO-NPs measured in soil solution do not seem to affect the NPs mobility in soil columns. Indeed in the loam soils, CuO-NPs breakthrough curves were very similar (Figure 2) although their zeta potential values were the most contrasted in the low and high OM soils (Figure 1).

All CuO-NPs breakthrough curves were well fitted with the CXTFIT code, using the hydrodynamic parameters determined with the water tracer. In all soils, the eluted CuO-NPs presented a fast transport in the columns without retardation as compared to the Br tracer (Table 4). In some soils, CuO-NPs presented a fast transport (faster than the tracer) indicating that these particles did not see all the water present in the soil, indicating some size exclusion processes during their transport, in agreement with the colloid filtration theory.

To the authors' knowledge, this is the first study on the transport of CuO-NPs in natural soils. We have shown that CuO-NPs could be partly mobile in very contrasted soils although the majority (86 to 94.2%) of these NPs are retained in the soils. Further work is needed to evaluate precisely the role of DOC in CuO-NPs transport, especially the influence of the type and concentration of natural OM present in the soil solutions.

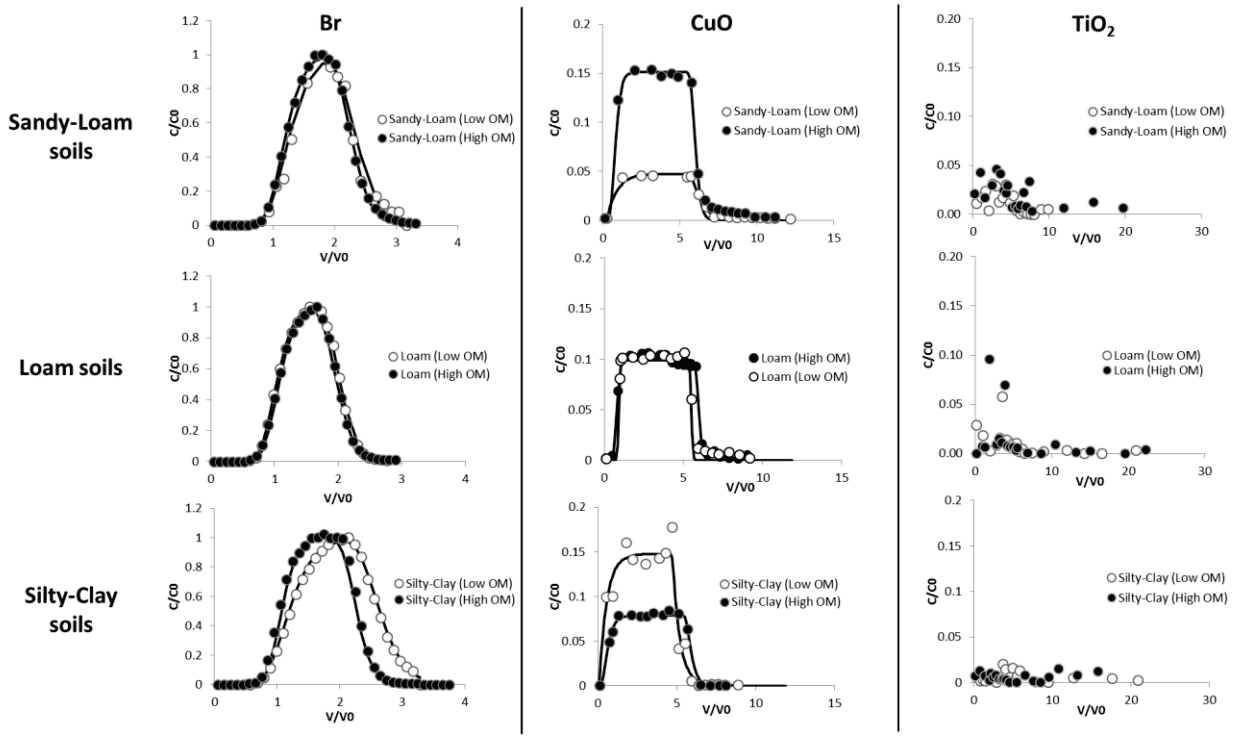


Figure 2 Breakthrough curves of Br, CuO and TiO_2 with CXTFIT predicted Br and CuO concentrations in columns of the 6 soils. Symbols represent the measured data, and black lines were calculated with the CXTFIT code by fitting with STANMOD.

Table 4 Transport parameters for bromide, CuO-NPs and TiO_2–NPs experiments.

		Bromide			CuO-NPs				TiO_2-NPs
		MR* (%)	D (cm^2/s)	R (-)	MR* (%)	D (cm^2/s)	R (-)	μ (h^{-1})	MR* (%)
Sandy-loam	**Low OM**	96.2	1.0E-07	1.01	5.2	1.35E-05	1.10	7.27	2.1
	High OM	99.1	7.1E-08	0.94	14.0	2.52E-07	1.01	1.97	5.5
Loam	**Low OM**	98.4	1.0E-07	0.96	11.4	1.10E-08	0.93	2.29	2.3
	High OM	99.0	5.9E-08	0.96	9.7	8.30E-08	0.87	2.37	3.4
Silty-clay	**Low OM**	101.3	1.2E-07	0.96	12.1	1.04E-05	1.56	2.37	1.9
	High OM	98.7	5.5E-08	0.82	6.8	9.47E-07	1.23	3.28	2.2

* mass recovery (%)

Transport of TiO_2-NPs in soil columns

The mobility of TiO_2-NPs in all soil columns was found to be very low with mass recoveries ranging between 1.5 and 5.5% (Table 4). In the 6 soils, the breakthrough curves were noisy indicating that the measured Ti concentrations were close to Ti background concentration in the leachates (Figure 2). Consequently, no transport modelling was conducted on TiO_2-NPs breakthrough curves. In all soils, TiO_2-NPs retention was higher than CuO-NPs. This very strong retention probably relates to the higher homoaggregation of TiO_2-NPs in soil solutions (Figure 1) (Vitorge *et al.*, 2013). Contrary to CuO-NPs, no clear influence of OM content on TiO_2-NPs mobility was noticed in the 6 soils. The high retention of TiO_2-NPs in soil is consistent with previous studies (Nickel *et al.*, 2015; Wang *et al.*, 2014) although these experiments were performed with positively charged TiO_2-NPs. As in the present work, Fang *et al.,* (2009) used negatively charged TiO_2-NPs and observed a significant transport of these NPs, especially in soils with low clay content and ionic strength. Since the retention of TiO_2-NPs was very high in the 6 soils of this study, it was not possible to observe a clear influence of the soil clay content (soil texture) or of the ionic strength. The differences with the results of Fang *et al.,* (2009) could be due to the different methodology used to assess TiO_2-NPs transport, especially the NPs input concentration, the column size and setup and the titanium measurement technique in the effluents. Studying the fate of TiO_2-NPs in soil is very challenging as Ti is very abundant in soils and has a heterogeneous distribution. The findings obtained with saturated soil columns carried out in laboratory conditions give a first assessment of the risk of NPs migration to deeper soil layers or groundwater. However, further work is needed to determine the transport potential of NPs with environmentally relevant concentrations, in undisturbed soils or under unsaturated conditions, in order to be closer to expected field conditions (Duester *et al.*, 2011; Cornelis *et al.*, 2014).

ACKNOWLEDGMENTS

Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region – ARC Environnement. This work was funded by a grant from the French National Program Microbien EC2CO-CNRS. The authors thank the technical assistance of Erwann Vince and Sophie Sebastianutti (MOME platform, Envirhonalp).

REFERENCES

Badawy AME, Luxton TP, Silva RG, Scheckel KG, Suidan MT, Tolaymat TM. (2010). Impact of Environmental Conditions (pH, Ionic Strength, and Electrolyte Type) on the Surface Charge and Aggregation of Silver Nanoparticles Suspensions. *Environ Sci Technol* **44**:1260–1266.

Ben-Moshe T, Dror I, Berkowitz B. (2010). Transport of metal oxide nanoparticles in saturated porous media. *Chemosphere* **81**:387–393.

Chen X, Mao SS. (2007). Titanium Dioxide Nanomaterials: Synthesis, Properties, Modifications, and Applications. *Chem Rev* **107**:2891–2959.

Cornelis G, Hund-Rinke K, Kuhlbusch T, Brink N van den, Nickel C. (2014). Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. *Critical Reviews in Environmental Science and Technology* **44**:2720–2764.

Cornelis G, Pang L, Doolette C, Kirby JK, McLaughlin MJ. (2013). Transport of silver nanoparticles in saturated columns of natural soils. *Science of The Total Environment* **463–464**:120–130.

Duester L, Prasse C, Vogel JV, Vink JPM, Schaumann GE. (2011). Translocation of Sb and Ti in an undisturbed floodplain soil after application of Sb2O3 and TiO2 nanoparticles to the surface. *J Environ Monit* **13**:1204–1211.

Fang J, Shan X, Wen B, Lin J, Owens G. (2009). Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. *Environmental Pollution* **157**:1101–1109.

Fang J, Shan X, Wen B, Lin J, Owens G, Zhou S. (2011). Transport of copper as affected by titania nanoparticles in soil columns. *Environmental Pollution* **159**:1248–1256.

French RA, Jacobson AR, Kim B, Isley SL, Penn RL, Baveye PC. (2009). Influence of Ionic Strength, pH, and Cation Valence on Aggregation Kinetics of Titanium Dioxide Nanoparticles. *Environ Sci Technol* **43**:1354–1359.

Jacobson AR, Dousset S, Andreux F, Baveye PC. (2007). Electron Microprobe and Synchrotron X-ray Fluorescence Mapping of the Heterogeneous Distribution of Copper in High-Copper Vineyard Soils. *Environ Sci Technol* **41**:6343–6349.

Keller AA, McFerran S, Lazareva A, Suh S. (2013). Global life cycle releases of engineered nanomaterials. *J Nanopart Res* **15**:1–17.

Lakshmanan S, Holmes WM, Sloan WT, Phoenix VR. (2015). Nanoparticle transport in saturated porous medium using magnetic resonance imaging. *Chemical Engineering Journal* **266**:156–162.

Martins JMF, Majdalani S, Vitorge E, Desaunay A, Navel A, Guiné V, *et al.* (2013). Role of macropore flow in the transport of Escherichia coli cells in undisturbed cores of a brown leached soil. *Environ Sci: Processes Impacts* **15**:347–356.

Martins JM, Mermoud A. (1998). Sorption and degradation of four nitroaromatic herbicides in mono and multi-solute saturated/unsaturated soil batch systems. *Journal of Contaminant Hydrology* **33**:187–210.

Morel M-C, Spadini L, Brimo K, Martins JMF. (2014). Speciation study in the sulfamethoxazole–copper–pH–soil system: Implications for retention prediction. *Science of The Total Environment* **481**:266–273.

Navel A, Martins JMF. (2014). Effect of long term organic amendments and vegetation of vineyard soils on the microscale distribution and biogeochemistry of copper. *Science of The Total Environment* **466–467**:681–689.

Nickel C, Gabsch S, Hellack B, Nogowski A, Babick F, Stintz M, *et al.* (2015). Mobility of coated and uncoated TiO2 nanomaterials in soil columns–Applicability of the tests methods of OECD TG 312 and 106 for nanomaterials. *Journal of environmental management* **157**:230–237.

Nowack B, Ranville JF, Diamond S, Gallego-Urrea JA, Metcalfe C, Rose J, *et al.* (2012). Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environmental Toxicology and Chemistry* **31**:50–59.

Pan B, Xing B. (2012). Applications and implications of manufactured nanoparticles in soils: a review. *European Journal of Soil Science* **63**:437–456.

Ren G, Hu D, Cheng EWC, Vargas-Reus MA, Reip P, Allaker RP. (2009). Characterisation of copper oxide nanoparticles for antimicrobial applications. *International Journal of Antimicrobial Agents* **33**:587–590.

Simonin M, Guyonnet JP, Martins JMF, Ginot M, Richaume A. (2015). Influence of soil properties on the toxicity of TiO2 nanoparticles on carbon mineralization and bacterial abundance. *Journal of Hazardous Materials* **283**:529–535.

Thio BJR, Zhou D, Keller AA. (2011). Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles. *Journal of Hazardous Materials* **189**:556–563.

Toride N, Leij FJ, Van Genuchten MT. (1995). The CXTFIT code for estimating transport parameters from laboratory and field tracer experiments.

Vitorge E, Szenknect S, Martins JMF, Barthès V, Auger A, Renard O, *et al.* (2014). Comparison of three labeled silica nanoparticles used as tracers in transport experiments in porous media. Part I: Syntheses and characterizations. *Environmental Pollution* **184**:605–612.

Vitorge E, Szenknect S, Martins JMF, Gaudet J-P. (2013). Size- and concentration-dependent deposition of fluorescent silica colloids in saturated sand columns: transport experiments and modeling. *Environ Sci: Processes Impacts* **15**:1590–1600.

Wang D, Su C, Zhang W, Hao X, Cang L, Wang Y, *et al.* (2014). Laboratory assessment of the mobility of water-dispersed engineered nanoparticles in a red soil (Ultisol). *Journal of Hydrology* **519, Part B**:1677–1687.

Wang Y, Gao B, Morales VL, Tian Y, Wu L, Gao J, *et al.* (2012). Transport of titanium dioxide nanoparticles in saturated porous media under various solution chemistry conditions. *J Nanopart Res* **14**:1–9.

Zhou D, Abdel-Fattah AI, Keller AA. (2012). Clay Particles Destabilize Engineered Nanoparticles in Aqueous Environments. *Environ Sci Technol* **46**:7520–7526.

Zhu J, Li D, Chen H, Yang X, Lu L, Wang X. (2004). Highly dispersed CuO nanoparticles prepared by a novel quick-precipitation method. *Materials Letters* **58**:3324–3327.

3. Influence des propriétés du sol sur l'impact du TiO_2 sur la respiration microbienne et l'abondance bactérienne

a. Article 3 : Présentation générale de l'étude et synthèse des principaux résultats

Compte tenu de la mobilité différente des NPs modèles en fonction du sol et de la forte rétention observée, nous avons souhaité évaluer (i) si les TiO_2-NPs présentaient une toxicité vis à vis des communautés microbiennes indigènes de ces sols et le cas échéant (ii) déterminer quelles propriétés des sols influençaient cette toxicité.

Les 6 sols ont donc été exposés à deux concentrations en TiO_2 (1 mg et 500 mg kg^{-1}) et incubés en microcosmes pendant 7, 30 et 90 jours. L'impact sur les communautés microbiennes du sol a été évalué en ciblant un processus microbien global du cycle du carbone : la respiration du sol, également appelée minéralisation du carbone, mesurée par la méthode dite de substrate-induced respiration (SIR). En parallèle, les effets sur l'abondance bactérienne ont été mesurés par PCR quantitative en ciblant le gène *16S ADNr* (également appelé *rrs*). Afin de déterminer l'influence des propriétés du sol sur les caractéristiques physico-chimiques des TiO_2-NPs, celles-ci ont été caractérisées dans les solutions de sol et dans de l'eau ultrapure en mesurant leur agrégation (diamètre hydrodynamique) et leur charge de surface (potentiel zêta) par Dynamic Light Scattering à l'aide d'un NanoZS (Malvern).

Aucun impact du TiO_2 n'a été constaté sur la minéralisation du carbone ou l'abondance bactérienne dans les sols, à l'exception du sol limono-argileux à forte teneur en MO. Dans ce sol, une diminution de la minéralisation du carbone a été observée dès 7 jours, même pour la dose faible de 1 mg kg^{-1} (jusqu'à -20%). Nos résultats suggèrent que la toxicité du TiO_2-NPs pourrait être liée au pH et à la teneur en MO mais pas à la teneur en argile des sols. De plus, nous avons constaté que dans la solution du sol limono-argileux (forte MO), les TiO_2-NPs seraient moins stables en suspension (potentiel zêta entre -15 mV et +15 mV), due à une diminution de la répulsion électrostatique. Cette caractéristique pourrait expliquer leur plus grande toxicité envers les microorganismes de ce sol, mais cela reste à vérifier.

Nos résultats indiquent que, dans la majorité des sols, le TiO_2 a un faible potentiel toxique envers les microorganismes. Dans l'unique sol où des effets délétères ont été observés, les

TiO_2-NPs avaient des propriétés physico-chimiques singulières par rapport aux autres sols, indiquant une plus faible stabilité en suspension.

b. « Influence of soil properties on the toxicity of TiO_2 nanoparticles on carbon mineralization and bacterial abundance"

L'article 3 intitulé «Influence of soil properties on the toxicity of TiO_2 nanoparticles on carbon mineralization and bacterial abundance » a été publié en 2015 dans *Journal of Hazardous Material*.

Journal of Hazardous Materials 283 (2015) 529–535

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Influence of soil properties on the toxicity of TiO_2 nanoparticles on carbon mineralization and bacterial abundance

Marie Simonin[a,b,c,d], Julien P. Guyonnet[a,b,c], Jean M.F. Martins[d], Morgane Ginot[a,b,c], Agnès Richaume[a,b,c,*]

[a] Université de Lyon, Lyon, France
[b] Université Claude Bernard Lyon 1, Villeurbanne, France
[c] CNRS, UMR 5557, Ecologie Microbienne, Université Lyon 1, Villeurbanne, France
[d] UJF-Grenoble/CNRS-INSU/G-INP/IRD, LTHE UMR 5564, Grenoble F-38041, France

HIGHLIGHTS

- We tested TiO_2-NPs effects on microbial communities in six contrasted soils.
- In all soils but one, TiO_2-NPs had no impact on microbial communities.
- A low dose of TiO_2-NPs decreases C-mineralization in a silty-clay soil.
- TiO_2-NPs toxicity is likely driven by soil pH and organic matter content.

ARTICLE INFO

Article history:
Received 16 May 2014
Received in revised form
27 September 2014
Accepted 3 October 2014
Available online 12 October 2014

Keywords:
TiO_2 nanomaterials
Microbial community
Microbial ecotoxicology
Realistic exposure

ABSTRACT

Information regarding the impact of low concentration of engineered nanoparticles on soil microbial communities is currently limited and the importance of soil characteristics is often neglected in ecological risk assessment. To evaluate the impact of TiO_2 nanoparticles (NPs) on soil microbial communities (measured on bacterial abundance and carbon mineralization activity), 6 agricultural soils exhibiting contrasted textures and organic matter contents were exposed for 90 days to a low environmentally relevant concentration or to an accidental spiking of TiO_2-NPs (1 and 500 mg kg^{-1} dry soil, respectively) in microcosms. In most soils, TiO_2-NPs did not impact the activity and abundance of microbial communities, except in the silty-clay soil (high OM) where C-mineralization was significantly lowered, even with the low NPs concentration. Our results suggest that TiO_2-NPs toxicity does not depend on soil texture but likely on pH and OM content. We characterized TiO_2-NPs aggregation and zeta potential in soil solutions, in order to explain the difference of TiO_2-NPs effects on soil C-mineralization. Zeta potential and aggregation of TiO_2-NPs in the silty-clay (high OM) soil solution lead to a lower stability of TiO_2-NP-aggregates than in the other soils. Further experiments would be necessary to evaluate the relationship between TiO_2-NPs stability and toxicity in the soil.

1. Introduction

Titanium dioxide nanoparticles (TiO_2-NPs) are engineered nanomaterials that are commonly used in diverse domains, such as cosmetics, sunscreens, paints, or coatings [1,2]. A large fraction of TiO_2-NPs in commercial products can reach natural ecosystems, especially the soil, which is one of the biggest sink for nanomaterials [3–5]. The major pathways for TiO_2-NPs to enter soil are through agricultural amendments of sewage sludge or after an accidental spill during industrial production [6,7]. The increasing environmental release of TiO_2-NPs raises concerns about the potential effect on soil functioning and consequently on its capacity to fulfill essential ecosystem services.

Soils are porous systems consisting in complex structured assemblies of mineral and organic particles combined with liquid and gaseous phases. Soil properties such as clay and organic matter (OM) contents or pH greatly influence the behavior and bioavailability of common pollutants like pesticides, heavy metals or polycyclic aromatic hydrocarbons [8–12]. However, little

* Corresponding author at: CNRS, UMR 5557, Ecologie Microbienne, Université Lyon 1, Villeurbanne, France. Tel.: +33 472432650; fax: +33 472431223.
E-mail address: agnes.richaume@univ-lyon1.fr (A. Richaume).

http://dx.doi.org/10.1016/j.jhazmat.2014.10.004
0304-3894/

information is available on the influence of soil properties on the aggregation and surface charge of NPs, which will determine their mobility and bioavailability [13–15].

Vittori Antisari et al. [16] demonstrated that metal oxide NPs are localized in small size (2–53 and <2 μm) soil aggregates indicating that such NPs interact essentially with clay minerals and OM. This preferential accumulation of NPs in the clay fraction suggests that NPs can be in contact with most of microbial communities inhabiting soil, since 40–70% of total soil bacteria are located in the 2–20 μm and <2 μm fractions [16,17].

Soil microorganisms are key players of many critical functions, such as biogeochemical cycling (e.g. carbon, nitrogen, sulphur and phosphorous cycles), plant productivity or climate regulation. Microbial communities are known to be sensitive ecological indicators of soil response to environmental perturbations [18–20] and can be good models to investigate TiO_2-NPs effect on soil functioning and quality [21]. Previous studies have shown that TiO_2-NPs can induce a decrease in microbial activity and a shift in bacterial community structure in soils [22–25]. However, these pioneer studies were performed only with high concentrations of TiO_2-NPs and usually with a single model soil. Since extrapolation of results from one contaminated soil to another is difficult because of the great heterogeneity of soils in terms of composition, structure and reactivity, studies conducted under more realistic concentrations and covering a wide range of soils are required.

In this study, we investigated TiO_2-NPs microbial ecotoxicity in 6 agricultural soils exhibiting contrasted texture (sandy-loam, loam and silty-clay) and organic matter content (low or high concentration). Since soil properties, such as texture or organic matter content, influence microbial community composition and the potential bioavailability of TiO_2-NPs, we expected contrasted TiO_2-NP toxicity in the different soils. We assessed the effects of TiO_2-NPs on soil microbial communities in a microcosm experiment, by measurements of carbon (C) mineralization activity and soil bacterial abundance using quantitative PCR. The soils were exposed to a low TiO_2-NPs concentration (1 mg kg^{-1} dry soil) in the range of the predicted TiO_2-NPs concentrations in soil [5] and a higher concentration representing an accidental pollution (500 mg kg^{-1} dry soil). In order to interpret and explain the differences of TiO_2-NPs effects on soil microbial communities, we characterized the apparent size and surface charge of TiO_2-NPs in soil solutions of the 6 agricultural soils using Dynamic Light Scattering (DLS).

2. Materials and methods

2.1. Soils

This study was conducted with soils belonging to three different textural classes: a sandy-loam, a loam and a silty-clay. Soils were collected from the upper 20 cm layer of 3 different agricultural sites located in the Rhône-Alpes and Burgundy regions of France. At each site, the sampling was performed in 2 plots, distant of less than 500 m with low and high OM contents. After collection, visible rocks, roots and plant litter were manually removed. The soil was sieved (2 mm) and homogenized before storage at 4 °C. The sandy-loam soil (Cambisol, WRB, 2006) was obtained at the SERAIL (Station Expérimentale Rhône-Alpes et Information Légumière), an agricultural research station at Brindas (Rhône, France) from a field under vegetable rotation. The high OM content soil was collected in a plot, which had been fertilized with organic matter (bark compost) since 1995. The low OM content soil was collected in another plot, which received no amendment during the same period. The loamy soil (Luvisol, WRB, 2006) was collected at La Côte Saint-André (Isère, France) in a field cropped with maize (low OM content) and under permanent pasture (high OM content). The silty-clay soil (Cambisol, WRB, 2006) was sampled at Commarin (Côte d'Or, France) from a field in rape–wheat–winter barley rotation (low OM content) and under an adjacent permanent pasture (high OM content). Soils were characterized (Table 1) by the Laboratoire d'analyse des sols (LAS, Arras, France), for particle-size analysis (textural class), OM content, cation exchange capacity (CEC) and titanium content. pH and ionic strength were measured according to ISO 10390 and ISO 11265 procedures. Main soil elements (Table S1) were also measured following US-EPA 3052 guidelines, using a microwave assisted (Novawave, SCP Science) acid digestion ($HF + HNO_3$). Element concentrations were determined using ICP-OES (Varian 700-ES).

2.2. Soil solutions

Soil solutions were prepared to characterize TiO_2-NPs in physicochemical conditions as close as possible to those encountered in soil in terms of pH, ionic strength and dissolved components. The protocol was designed to obtain the dissolved components present in soils after removal of soil colloids larger than the initial size of TiO_2-NPs (i.e. 20 nm) from the suspension. Dynamic light scattering (DLS) analysis does not allow differentiating the introduced TiO_2-NPs from the native nanometric soil components. The removal of possibly interfering soil components is thus a crucial step in the reliable measurement of TiO_2 NPs. Soil solutions were prepared by shaking 10 g of soil dispersed in 50 mL of ultrapure water (18 MΩ) during 30 min at 180 rpm and 20 °C in a refrigerated incubator shaker (New Brunswick – Eppendorf, Hamburg, Germany). The solutions were then centrifuged for 20 min at 8000 × g, 20 °C (Centrifuge 5804R, Eppendorf, Hamburg, Germany) to eliminate particles larger than 20 nm according to the Stokes' law. The supernatants were collected and stored at 4 °C. For each soils, solutions were prepared in triplicates. pH, ionic strength, cation concentrations (Ca, Fe, Na) using microwave assisted acid digestion and determined by ICP-OES (Varian 700-ES) and dissolved organic carbon (DOC) using a Shimadzu TOC-V (Shimadzu, Kyoto, Japan) were assessed in each soil solution (Table 1).

Table 1
Main properties of soils and soil solutions.

	Soils									Soil solutions					
	% Sand	% Loam	% Clay	% OM	WHC (%)	pH	Ionic strength (mM)	CEC (cmol$^+$ kg^{-1})	Ti (g kg^{-1})	pH	Ionic strength (mM)	DOC (mg L^{-1})	Ca (mg L^{-1})	Fe (mg L^{-1})	Na (mg L^{-1})
Sandy-loam – Low OM	68.4	14.7	16.9	2.09	20	7	0.98	11.5	2.44	6.7	1	11.6	21.7	1.45	8.89
Sandy-loam – High OM	65.6	16.1	18.3	4.46	20	6.9	1.59	15.4	2.38	6.4	2	18.6	12.2	1.69	12.1
Loam – Low OM	37.5	42.7	19.8	2.23	30	6.4	0.60	8.79	2.71	6.2	1.2	8.2	28.8	1.90	13.2
Loam – High OM	40.3	40.8	18.9	6.77	30	6.3	1.31	15.3	2.4	5.4	2.4	10.5	38.2	2.79	21.8
Silty-clay – Low OM	8.2	49.8	42.0	4.72	47	6.9	0.51	17.4	5.26	6.3	1.5	25.2	33.5	1.79	11.4
Silty-clay – High OM	10.1	50.8	39.1	7.87	51	7.7	1.37	20.1	4.52	7.1	1.6	8.9	80.8	3.53	5.48

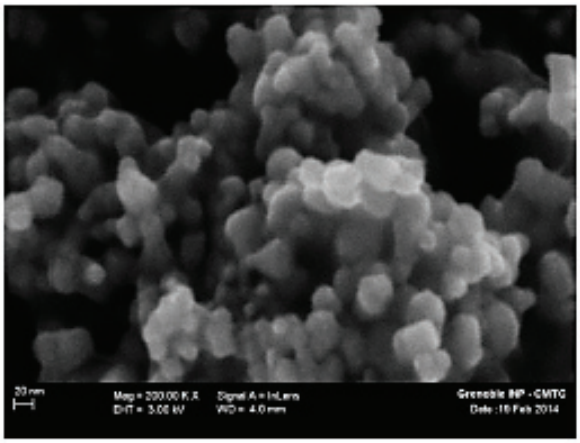


Fig. 1. Typical morphology of TiO_2 nanoparticles measured by SEM-FEG.

2.3. Nanoparticles characterization in ultrapure water and soil solutions

Titanium dioxide nanoparticles (TiO_2-NPs) were provided by Sigma–Aldrich (St Louis, USA) as a mixture of anatase (80%) and rutile (20%) crystal structure with at least 99.5% purity. According to the manufacturer information, TiO_2-NPs presented a specific surface area of 35–65 $m^2 g^{-1}$ and a mean particle size of 21 nm in powder as measured by Transmission Electron Microscopy. The particle size of TiO_2-NPs was verified (Fig. 1) using a ZEISS Ultra 55 scanning electron microscopy–field emission gun (SEM-FEG) and energy dispersive spectroscopy (EDS) with a SDD detector (BRUKER AXS-30 mm^2). TiO_2-NPs were dispersed in ultrapure water and a droplet of the suspensions was deposited on an aluminum support and allowed to evaporate at room temperature. The samples were then metalized with carbon using thermal vaporization in a Gatan apparatus (PECS 682). The average particle size of TiO_2-NPs measured was 28.7 nm.

The hydrodynamic diameter and zeta potential of TiO_2-NPs in water or soil solutions were determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern Instruments – UK). The surface charge was measured by electrophoretic mobility measurements converted to zeta potential and the particle size is presented as hydrodynamic diameter. TiO_2-NPs suspensions (100 mg L^{-1}) were prepared in ultrapure water or in soil solutions. All TiO_2-NPs suspensions were dispersed through ultrasonication for 5 min before use to ensure suspensions homogeneity. For each sample, the mean of 3 measurements was recorded. To determine the zero point of charge (ZPC) of TiO_2-NPs suspended in ultrapure water or in soil solutions, measurements of zeta potential were done at pH varying from 3 to 8 using an MPT-1 automatic titrator (Malvern Instrument) (HNO_3 (0.1 M) or NaOH (0.1 M) additions). For each measurement, control samples without TiO_2-NPs were analyzed to ensure that soil colloids did not interfere with the NPs analysis.

2.4. Soil microcosms and experimental design

One kg of soil (equivalent dry weight – eq dw) was spiked with a volume of TiO_2-NPs suspensions prepared in ultrapure water at 20 mg or 10 g TiO_2 L^{-1} in order to obtain 1 mg TiO_2 kg^{-1} or 500 mg TiO_2 kg^{-1} dry soil respectively and to increase the soil water content to the water holding capacity (WHC). The exposure doses were chosen to represent a low realistic environmental concentration (1 mg kg^{-1} dry soil) [5] and a high concentration representing an accidental spill (500 mg kg^{-1} dry soil). The TiO_2-NPs suspensions were added homogeneously using a multichannel pipette and then soils were thoroughly mixed for 10 min to ensure a uniform spiking. Soils receiving only the same volume of ultrapure water were used as controls. Microcosms were set up by placing 50 g of control or spiked soils (eq dw) into 150 mL glass plasma flasks sealed with rubber stoppers to avoid soil drying and to maintain the soil moisture constant during the experiment. Microcosms were incubated for 0, 7, 30 and 90 days in the dark at 28 °C. Microcosms were weekly aerated for 5 min to ensure a renewal of the atmosphere of the flasks. This experimental design resulted in a total of 324 microcosms with 6 replicates per treatment (6 soils × 3 concentrations of TiO_2-NPs × 3 sampling times × 6 replicates). At the beginning of the experiment, 36 additional microcosms (6 soils × 6 replicates) were prepared to measure the baseline conditions (T = 0 day) of the soil samples.

At the end of each incubation time, microcosms were subsampled as follows: 10 g of soil (eq dw) were immediately used for measurements of C-mineralization, 3 g of soil was stored at −20 °C before DNA extraction. The remaining samples were kept at 4 °C for further soil physicochemical analysis. Soil pH was measured after incubation with NPs in every soil microcosms according to the procedure cited above. Soil pH was not affected by the addition of TiO_2-NPs at the 3 sampling times (data not shown).

2.5. C-mineralization

Substrate induced respiration (SIR) rate, i.e. C-mineralization potential was measured according to Patra et al. [26]. Fresh soil collected immediately after sampling (10 g eq dw) was placed in a 150 mL plasma flask. Distilled water (0.5 mL) containing glucose was added to achieve a final concentration of 1.2 mg C-glucose g^{-1} dry soil. The flasks were sealed with rubber stoppers and incubated at 28 °C for 6 h. After 2 h of initial incubation, gas samples were analyzed every hour during the incubation to measure the increase in CO_2 emission using a gas chromatograph (Micro GC R3000, SRA Instrument, Marcy L'Etoile, France). C-mineralization was expressed as µg C-CO_2 h^{-1} g^{-1} dry soil.

2.6. DNA extraction and quantification

DNA was extracted from 0.5 g of frozen soil using the Power Soil™ DNA Isolation Kit (MO BIO laboratories, Carlsbad, CA, USA), following the manufacturer's instructions and then quantified using the Quant it™ Picogreen© dsDNA Assay kit (Molecular Probes, USA). Fluorescence was measured with a UV spectrophotometer Xenius (Safas, Monaco) (λ = 520 nm).

2.7. Quantification of the abundance of soil total bacteria

The abundance of soil total bacteria was measured by quantitative PCR targeting universal gene *rrs* coding for *16S* rRNA. Amplification was performed using gene primers 519F and 907R [27,28].

The final reaction volume was 20 µL and contained (final concentrations): 0.3 µM of each primer, 1X of Lightcycler 480 Probes Master Mix (Roche Diagnostics, Meylan, France) and 5 ng of soil DNA extract or 10^8–10^2 *16S* copy number of the pQuantAlb plasmid [29]. The amplification program was 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 1 min at 63 °C and 30 s at 72 °C and finally 10 s at 40 °C. Melting curves analysis confirmed the specificity of the amplification. All quantitative PCR reactions including unknown samples and standard curves were performed in duplicate.

2.8. Statistical analysis

All results are presented as means (± standard error). For each soil type, a one-way analysis of variance (ANOVA) and post hoc Tukey HSD were performed to test the effect of TiO_2-NPs concentration on measured variables at each sampling time. When necessary, data were log-transformed prior to analysis to ensure conformity with the assumptions of normality and homogeneity of variances. We also conducted multiple regression to identify the drivers of C-mineralization in soils. The TiO_2-NPs concentration and every soil parameters that were significantly related to C-mineralization were kept in the final model. Effects with $P < 0.05$ are referred to as significant. All statistical analysis was carried out using R statistical software 2.13.2 [30].

3. Results

3.1. Effect of TiO_2-NPs on C-mineralization

C-mineralization was not affected by the addition of TiO_2-NPs in most of the soils tested (sandy-loam – low and high OM; loam – high OM; silty-clay – low OM) at the 3 sampling dates (Fig. 2). However, after 7 days, a decrease of C-mineralization was observed in the loam soil (low OM) for the dose 1 (−22.7%, $P = 0.008$, Fig. 2A) but this effect appeared transitory and was no longer observed at 30 and 90 days of incubation. In the silty-clay soil (high OM) a significant decrease of C-mineralization over time was observed with both TiO_2-NPs concentrations (Fig. 2).

However, no clear dose-response was noticed since both concentrations induced approximately the same decrease of C-mineralization after 7 days (dose 1: −19.8%, $P = 0.01$; dose 500: −21.2%, $P = 0.006$) and 30 days (dose 1: −14.2%, $P = 0.02$; dose 500: −12.1%, $P = 0.05$). After 90 days, a decrease was observed only for the dose 500 (−15.9%, $P = 0.001$), whereas a trend to resiliency occurred for the dose 1 as shown in Fig. 3.

3.2. Effect of TiO_2-NPs on bacterial abundance

In most cases, the bacterial abundance was not significantly affected by the presence of TiO_2-NPs (Table 2), except in the loamy soil (low OM) 7 days after spiking with 500 mg kg^{-1} (−25%, $P = 0.03$). In the silty-clay soil (high OM), decreases of 24% and 37% were observed after 90 days for the TiO_2-NPs doses 1 and 500, respectively. However, these decreases were not significant because of high variability in the quantitative PCR data (Table 2).

3.3. TiO_2-NPs characteristics in water and soil solutions

The average size of TiO_2-NPs measured by DLS in ultrapure water was 158 nm (±7.2) (Fig. 4). In control soil solutions (without TiO_2-NPs), colloid sizes did not exceed 28.9 nm (±3.3), consistently to what was expected according to the Stokes law (Fig. 4).

When TiO_2-NPs were added to soil solutions, the peak corresponding to the size of the natural soil colloids (around 20 nm) was no longer detected, indicating that their relative abundance were negligible compared to that of added TiO_2-NPs (100 mg L^{-1}) (data not shown). In soil solutions, TiO_2-NPs were always significantly less aggregated (smaller apparent size) than in ultrapure water except in the solution of the silty-clay soil (high OM) (Fig. 4). The sizes of TiO_2-NPs measured in the soil solutions were not significantly different except between the two silty-clay soil solutions, with size values ranging from 74 nm (±14.9) in the silty-clay soil with low OM content to 128 nm (±21.7) in the silty-clay soil with high OM content (Fig. 4). When dispersed in ultrapure water, TiO_2-NPs presented a zeta potential of - 13.4 mV (±0.5) and a zero point

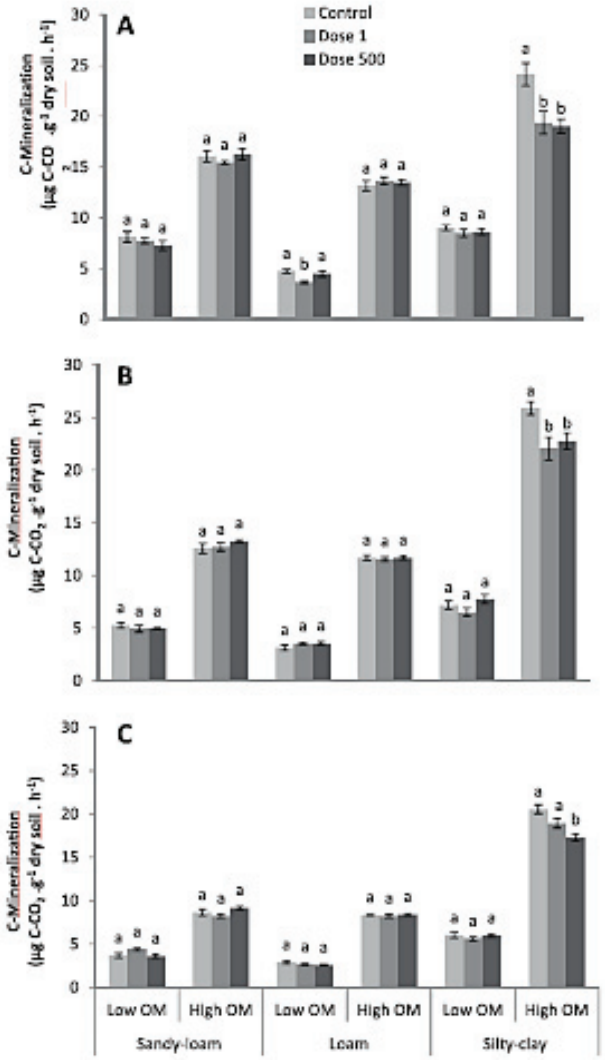


Fig. 2. C-mineralization in the 6 soils after (A) 7, (B) 30 and (C) 90 days in the different treatments (Control, Dose 1, Dose 500 mg kg^{-1} TiO_2-NPs). Means and standard errors are presented ($n = 6$). For each soil, bars labeled with the same letter do not differ at $P < 0.05$.

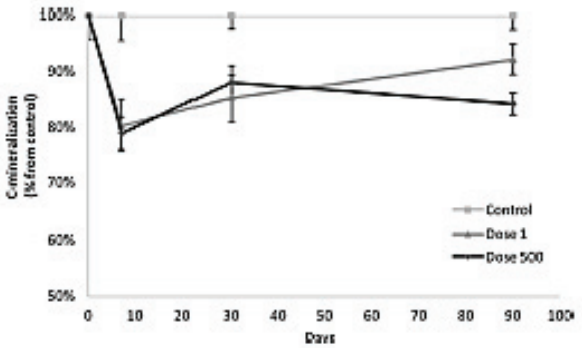


Fig. 3. Changes in C-mineralization in the silty-clay soil (high OM) in the different treatments (Control, Dose 1, Dose 500 mg kg^{-1} TiO_2-NPs). Data are expressed as the percentage change from the control treatment. Means and standard errors are presented ($n = 6$).

Table 2
Bacterial abundance ($\times 10^9$ *rrs* copy number g^{-1} dry soil) in the different TiO_2-NPs treatments (Control, Dose 1, Dose 500 mg kg^{-1} TiO_2-NPs) after 7, 30 and 90 days. Means ± standard errors are presented (n = 6).

	7 days			30 days			90 days		
	Control	Dose 1	Dose 500	Control	Dose 1	Dose 500	Control	Dose 1	Dose 500
Sandy-loam – Low OM	5.85 ± 0.3	5.91 ± 0.4	6.56 ± 0.2	3.07 ± 0.7	3.46 ± 0.5	2.62 ± 0.6	n.d	n.d	n.d
Sandy-loam – High OM	5.14 ± 0.3	5.09 ± 0.5	6.51 ± 0.5	4.08 ± 0.6	4.02 ± 0.3	3.64 ± 0.4	n.d	n.d	n.d
Loam – Low OM	5.77 ± 0.4	4.84 ± 0.3	**4.32 ± 0.3**	3.3 ± 1.1	3.3 ± 1.2	2.82 ± 0.7	n.d	n.d	n.d
Loam – High OM	7.43 ± 1.6	6.76 ± 0.3	6.25 ± 1.2	6.94 ± 1.0	6.31 ± 1.0	6.61 ± 0.9	n.d	n.d	n.d
Silty-clay – Low OM	7.69 ± 1.6	5.38 ± 1.3	7.08 ± 1.1	5.30 ± 1.8	3.44 ± 1.0	5.30 ± 1.5	n.d	n.d	n.d
Silty-clay – High OM	38.2 ± 5	32 ± 7	31.7 ± 4	33.1 ± 4	30.4 ± 5	31.5 ± 3	29.9 ± 4	22.8 ± 5	18.3 ± 2

Value in bold is significant ($P < 0.05$); n.d is not determined.

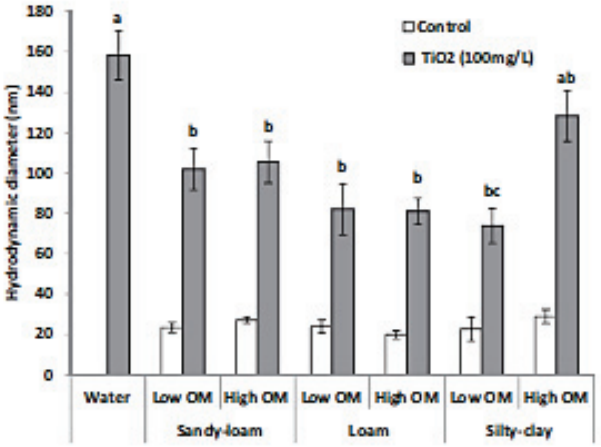


Fig. 4. Hydrodynamic diameters of TiO_2-NPs (100 mg TiO_2-NPs L^{-1}) measured in ultrapure water and soil solutions by dynamic light scattering (DLS). Controls are soils solutions without TiO_2-NPs added. Means and standard errors are presented (n = 3). Means followed by the same letter are not significantly different at $P < 0.05$.

of charge at pH 5.5 (Fig. 5). In contrast, when dispersed in soils solutions, TiO_2-NPs were always negatively charged whatever the pH (Fig. 5).

4. Discussion

TiO_2-NPs can be toxic toward bacteria through membrane disorganization and reactive oxygen species (ROS) production due

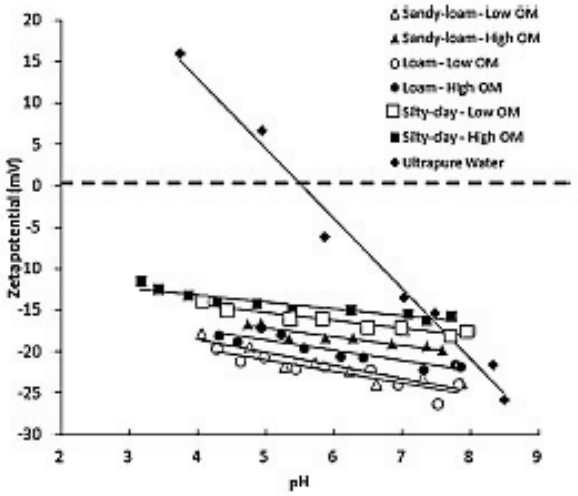


Fig. 5. Zeta potential of TiO_2-NPs in function of pH measured in ultrapure water and soil solutions by DLS.

to their adsorption to cell membrane and photocatalytic oxidation [31,32]. However, in most soils of the present study, TiO_2-NPs had no significant effect on either C-mineralization potential activity or on bacterial abundance, whatever the concentration (1 or 500 mg kg^{-1} dry soil) over 90 days. The C-mineralization and bacterial abundance values were in the range of other studies carried out with arable or grassland soils [23,26,33,34]. A decrease of C-mineralization with 500 mg kg^{-1} of TiO_2-NPs over 60 days has been reported in a loam soil [23]. In the present study, in both loam soils tested, the same concentration had no effect, except a slight and transitory decrease of bacterial abundance after 7 days. Using another metal NP, Pawlett et al. [35] reported that the impact of zero-valent iron NPs on C-mineralization was highly soil-dependant. Only the silty-clay soil (high OM) was strongly affected by TiO_2-NPs contamination. The absence of negative effects in the silty-clay (low OM) soil suggests that soil clay content does not influence TiO_2-NPs toxicity. Conversely, TiO_2-NPs toxicity may be related to soil OM content and pH, as the negative effects on C-mineralization were only observed in the soil exhibiting the highest pH and OM content. These assumptions are supported by a multiple regression analysis (Table S2) that highlights a significant interaction between TiO_2-NPs effects and pH or OM content, but not with clay content. Further soil experiments specifically designed to test a wider range of OM content and pH are needed to generalize the role of these parameters in TiO_2-NPs toxicity.

The toxicity of NPs is known to be influenced by their aggregation state and stability [32,36], which are controlled by environmental factors. The role of OM as a dispersing and stabilizing agent of TiO_2-NPs in aqueous media has been reported [37,38]. Consistently, TiO_2-NPs appeared more dispersed in soil solutions than in ultrapure water in the present work. This could be due to the interactions of dissolved OM and NPs that are known to change the NPs surface properties and/or increase steric repulsions [39,40]. The aggregation of TiO_2-NPs was measured and compared between the different soil solutions exhibiting contrasted physicochemical properties. No significant differences were observed, except in the silty-clay soil with low OM and high OM content. In the silty-clay (high OM) soil solution, TiO_2-NPs were the most aggregated. In the latter case, the size of NPs aggregates was not significantly different from the size measured in ultrapure water. The low concentration of DOC in this soil solution could explain the similarity of TiO_2-NPs aggregation in this soil and in pure water. Another explanation could be the high concentration of Ca in relation with the alkaline pH of this soil. Indeed it has been demonstrated that aggregation of NPs increases in presence of divalent cations [37,41].

The ionic strength is also a key environmental factor for NPs properties. As observed in this study, it is normally less than 0.005 M in soil solution [42]. Consistently with the literature, the ionic strength of soil solutions was positively correlated with the zeta potential of TiO_2-NPs ($R^2 = 0.50$, $P = 0.01$, $n = 18$) [43,44]. The changes of NPs zeta potential with ionic strength are the result of

electrostatic double layer compression by charge screening [43]. As previously observed by Zhang et al. [44], the zeta potential of TiO_2-NPs measured in the 6 soils solutions was always negative in presence of natural OM, within the tested pH range. At the opposite, in ultrapure water TiO_2-NPs presented a ZPC at pH = 5.5, which is consistent with the literature [40,45,46]. The negative zeta potential of TiO_2-NPs in soil solutions may be due to the imparting of negative charges from OM to NPs surfaces [44]. Moreover a positive correlation between zeta potential and iron was observed ($R^2 = 0.43$, $P = 0.02$, $n = 18$), emphasizing the potential key role of cations on NPs properties. The increase of zeta potential with cation concentration has been explained by the neutralization of the negative charge that OM imparted to NPs [44].

The strongest effects of TiO_2-NPs on C-mineralization were observed in the silty-clay soil (high OM). In the corresponding soil solution, TiO_2-NPs were less negatively charged and more aggregated than in the others, due to the high ionic strength, the high Ca concentration and the low DOC content. Fang et al. [47] found a positive correlation between TiO_2-NPs stability in soil suspensions with DOC content and a negative correlation was observed with ionic strength, pH and zeta potential. Based on these results, it is likely that TiO_2-NPs in the silty-clay (high OM) soil solution exhibit properties conferring low stability in suspension. More research are needed to determine whether the impact of TiO_2-NPs on microbial activity in the silty-clay soil (high OM) can be related to the instability of TiO_2-NPs aggregates or not. Microscale studies of the interactions between microbes, NPs and soil components would be very useful to evaluate the relationship between the TiO_2-NPs stability and toxicity in soil.

Besides the role of the physicochemical NPs properties, the absence of TiO_2-NPs effect on C-mineralization in most of the soils could also depend on the composition of the indigenous microbial community, which may be more resistant to such NPs perturbation than the silty-clay soil with high OM content. Future research should address the role of soil microbial diversity in the response to TiO_2-NPs toxicity to clarify the impact on soil functioning.

Only few studies have investigated the effect of low concentrations of NPs on soil microbial communities [16,49,50]. To our knowledge, this is the first work evaluating the impact of such a low concentration of TiO_2-NPs on soil microorganisms. By using two doses of NPs, we expected to observe a dose-response of TiO_2-NPs that would lead to a higher decrease of C-mineralization with 500 mg kg^{-1} than with 1 mg kg^{-1}. This was not observed here, as the low concentration induced similar or even higher negative effects than a 500-fold higher concentration (Fig. 2A and B). The absence of a clear dose-response on C-mineralization has already been reported by Ge at al. [23] for 0.5–2 g of TiO_2-NPs kg^{-1}. These results are likely due to NPs homo- and hetero-aggregation processes, which are controlled by the NPs concentrations applied to soil [48], explaining the absence of linear dose-response observed in soil.

5. Conclusion

This work shows that a low concentration of TiO_2-NPs can have detrimental effects on microbial mineralization in soil. However, contrasted responses to TiO_2-NPs spiking were observed among the six studied soils. Contrary to what is observed with common pollutants (such as heavy metals or polycyclic aromatic hydrocarbon) that cause more toxic effect in coarse-textured soils [8,51], TiO_2-NPs induced a lasting decrease of microbial activity only in a silty-clay soil, whereas sandy soils were not affected. It seems that soil texture is not a crucial factor in TiO_2-NPs toxicity. Among the multiple environmental factors, pH and OM content might be the major drivers that affect NPs toxicity in soil. Although not reflecting exactly the physicochemical conditions encountered in soil, the characterization of TiO_2-NPs properties in soil solutions allowed to propose hypotheses explaining these results. We assume that the properties of TiO_2-NPs measured in the silty-clay (high OM) soil solution lead to a lower stability of NPs aggregates that might explain their higher toxicity to microorganisms than in the other soils.

Altogether, our results showed that the use of low concentrations of NPs coupled with their characterization directly in solutions of a wide range of natural soils provided useful information for ecotoxicological studies and appeared to be a relevant step toward a better understanding of the impact of NPs on soil functioning.

Acknowledgments

Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region – ARC Environnement. This work was funded by a grant from the French National Program Microbien EC2CO-CNRS. The authors thank the lab assistance of Pauline Bazoge, Florian Vautrin and Erwann Vince (MOME platform, Envirhonalp). We are grateful to Sylvie Nazaret and Alain Hartmann for their help during soil collection. C-mineralization measurements were performed at the AME platform (Microbial Ecology UMR5557-USC1364, Lyon) and quantitative PCR at the DTAMB platform (IFR 41, University Lyon 1).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.10.004.

References

[1] A. Boxall, Q. Chaudhry, C. Sinclair, A. Jones, R. Aitken, B. Jefferson, C. Watts, Current and Future Predicted Environmental Exposure to Engineered Nanoparticles, Central Science Laboratory, Department of the Environment and Rural Affairs, London, UK, 2007.
[2] C.O. Robichaud, A.E. Uyar, M.R. Darby, L.G. Zucker, M.R. Wiesner, Estimates of upper bounds and trends in nano-TiO_2 production as a basis for exposure assessment, Environ. Sci. Technol. 43 (2009) 4227–4233.
[3] F. Gottschalk, T. Sonderer, R.W. Scholz, B. Nowack, Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions, Environ. Sci. Technol. 43 (2009) 9216–9222.
[4] A.A. Keller, S. McFerran, A. Lazareva, S. Suh, Global life cycle releases of engineered nanomaterials, J. Nanopart. Res. 15 (2013) 1692.
[5] T.Y. Sun, F. Gottschalk, K. Hungerbühler, B. Nowack, Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials, Environ. Pollut. 185 (2014) 69–76.
[6] S.K. Brar, M. Verma, R. Tyagi, R. Surampalli, Engineered nanoparticles in wastewater and wastewater sludge – evidence and impacts, Waste Manage. 30 (2010) 504–520.
[7] M. Kiser, P. Westerhoff, T. Benn, Y. Wang, J. Perez-Rivera, K. Hristovski, Titanium nanomaterial removal and release from wastewater treatment plants, Environ. Sci. Technol. 43 (2009) 6757–6763.
[8] K.E. Giller, E. Witter, S.P. Mcgrath, Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils, a review, Soil Biol. Biochem. 30 (1998) 1389–1414.
[9] L. Jocteur Monrozier, P. Guez, A. Chalamet, R. Bardin, J.M.F. Martins, J.P. Gaudet, Distribution of microorganisms and fate of xenobiotic molecules in unsaturated soil environments, Sci. Total Environ. 136 (1993) 121–133.
[10] V. Labud, C. Garcia, T. Hernandez, Effect of hydrocarbon pollution on the microbial properties of a sandy and a clay soil, Chemosphere 66 (2007) 1863–1871.
[11] J.M.F. Martins, A. Mermoud, Sorption and degradation of four nitroaromatic herbicides in mono and multi-solute saturated/unsaturated soil batch systems, J. Contam. Hydrol. 33 (1998) 187–210.
[12] L. Ranjard, S. Nazaret, F. Gourbière, J. Thioulouse, P. Linet, A. Richaume, A soil microscale study to reveal the heterogeneity of Hg (II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints FEMS, Microbiol. Ecol. 31 (2000) 107–115.
[13] B. Pan, B.S. Xing, Applications and implications of manufactured nanoparticles in soils, a review, Eur. J. Soil Sci. 63 (2012) 437–456.
[14] G. Cornelis, L. Pang, C. Doolette, J.K. Kirby, M.J. McLaughlin, Transport of silver nanoparticles in saturated columns of natural soils, Sci. Total Environ. 463 (2013) 120–130.

[15] E. Vitorge, S. Szenknect, J.M.F. Martins, V. Barthes, J-P. Gaudet, Comparison of three labeled silica nanoparticles used as tracers in transport experiments in porous media. Part II. Transport experiments and modeling, Environ. Pollut. 184 (2014) 605–612.

[16] L. Vittori Antisari, S. Carbone, A. Gatti, G. Vianello, P. Nannipieri, Toxicity of metal oxide (CeO_2, Fe_3O_4, SnO_2) engineered nanoparticles on soil microbial biomass and their distribution in soil, Soil Biol. Biochem. 60 (2013) 87–94.

[17] L. Ranjard, A. Richaume, Quantitative and qualitative microscale distribution of bacteria in soil, Res. Microbiol. 152 (2001) 707–716.

[18] F. Baptist, L.Zinger, J.-C. Clement, C. Gallet, R. Guillemin, J.M.F. Martins, L. Sage, B. Shahnavaz, P. Choler, R. Geremia, Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach, Environ. Microbiol. 10 (2008) 799–809.

[19] D.P. Lejon, J.M.F. Martins, J. Lévêque, L. Spadini, N. Pascault, D. Landry, M-J. Milloux, V. Nowak, R. Chaussod, L. Ranjard, Copper dynamics and impact on microbial communities in soils of variable organic status, Environ. Sci. Technol. 42 (2008) 2819–2825.

[20] M. Schloter, O. Dilly, J. Munch, Indicators for evaluating soil quality, Agric. Ecosyst. Environ. 98 (2003) 255–262.

[21] P.A. Holden, J.P. Schimel, H.A. Godwin, Five reasons to use bacteria when assessing manufactured nanomaterial environmental hazards and fates, Curr. Opin. Biotechnol. 27 (2014) 73–78.

[22] W.C. Du, Y.Y. Sun, R. Ji, J.G. Zhu, J.C. Wu, H.Y. Guo, TiO_2 and ZnO nanoparticles negatively affect wheat growth and soil enzyme activities in agricultural soil, J. Environ. Monit. 13 (2011) 822–828.

[23] Y.G. Ge, J.P. Schimel, P.A. Holden, Evidence for negative effects of TiO_2 and ZnO nanoparticles on soil bacterial communities, Environ. Sci. Technol. 45 (2011) 1659–1664.

[24] Y.G. Ge, J.P. Schimel, P.A. Holden, Identification of soil bacteria susceptible to TiO_2 and ZnO nanoparticles, Appl. Environ. Microbiol. 78 (2012) 6749–6758.

[25] V. Nogueira, I. Lopes, T. Rocha-Santos, A.L. Santos, G.M. Rasteiro, F. Antunes, F. Gonçalves, A.M.V.M. Soares, A. Cunha, A. Almeida, N.N.C.M. Gomes, R. Pereira, Impact of organic and inorganic nanomaterials in the soil microbial community structure, Sci. Total Environ. 424 (2012) 344–350.

[26] A.K. Patra, L. Abbadie, A. Clays-Josserand, V. Degrange, S.J. Grayston, P. Loiseau, X. Le Roux, Effects of grazing on microbial functional groups involved in soil N dynamics, Ecol. Monogr. 75 (2005) 65–80.

[27] D.J. Lane, 16S/23S rRNA sequencing, in: E. Stackebrandt, M. Goodfellow (Eds.), Nucleic Acid Techniques in Bacterial Systematics, John Wiley and Sons, Chichester, 1991, pp. 115–175.

[28] G. Muyzer, A. Teske, C.O. Wirsen, H.W. Jannasch, Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments, Arch. Microbiol. 164 (1995) 165–172.

[29] P. Tortosa, A. Courtiol, S. Moutailler, A.B. Failloux, M. Weill, Chikungunya-Wolbachia interplay in *Aedes albopictus*, Insect. Mol. Biol. 17 (2008) 677–684.

[30] R Development Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2011, ISBN 3-900051-07-0, URL http://www.R-project.org/

[31] L.K. Adams, D.Y. Lyon, P.J. Alvarez, Comparative eco-toxicity of nanoscale TiO_2, SiO_2, and ZnO water suspensions, Water Res. 40 (2006) 3527–3532.

[32] A.L. Simon-Deckers, S. Loo, M. Mayne-L'hermite, N. Herlin-Boime, N. Menguy, C.C. Reynaud, B. Gouget, M. Carrière, Size-, composition-and shape-dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria, Environ. Sci. Technol. 43 (2009) 8423–8429.

[33] S. Henry, D. Bru, B. Stres, S. Hallet, L. Philippot, Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils, Appl. Environ. Microbiol. 72 (2006) 5181–5189.

[34] D. Roux-Michollet, S. Czarnes, B. Adam, D. Berry, C. Commeaux, N. Guillaumaud, A. Clays-Josserand, Effects of steam disinfestation on community structure, abundance and activity of heterotrophic, denitrifying and nitrifying bacteria in an organic farming soil, Soil Biol. Biochem. 40 (2008) 1836–1845.

[35] M. Pawlett, K. Ritz, R.A. Dorey, S. Rocks, J. Ramsden, J.A. Harris, The impact of zero-valent iron nanoparticles upon soil microbial communities is context dependent, Environ. Sci. Pollut. Res. 20 (2012) 1041–1049.

[36] L.R. Pokhrel, B. Dubey, P.R. Scheuerman, Natural water chemistry (dissolved organic carbon, pH, and hardness) modulates colloidal stability, dissolution, and antimicrobial activity of citrate functionalized silver nanoparticles, Environ. Sci. Nano 1 (2014) 45–54.

[37] R.A. French, A.R. Jacobson, B. Kim, S.L. Isley, R.L. Penn, P.C. Baveye, Influence of ionic strength, pH, and cation valence on aggregation kinetics of titanium dioxide nanoparticles, Environ. Sci. Technol. 43 (2009) 1354–1359.

[38] A.A. Keller, H. Wang, D. Zhou, H.S. Lenihan, G. Cherr, B.J. Cardinale, R. Miller, Z. Ji, Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices, Environ. Sci. Technol. 44 (2010) 1962–1967.

[39] R.F. Domingos, N. Tufenkji, K.J. Wilkinson, Aggregation of titanium dioxide nanoparticles, role of a fulvic acid, Environ. Sci. Technol. 43 (2009) 1282–1286.

[40] B.J.R. Thio, D.Z.hou, A.A. Keller, Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles, J. Hazard. Mater. 189 (2011) 556–563.

[41] A.M.E. Badawy, T.P. Luxton, R.G. Silva, K.G. Scheckel, M.T. Suidan, T.M. Tolaymat, Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions, Environ. Sci. Technol. 44 (2010) 1260–1266.

[42] A.S. Black, A.S. Campbell, Ionic strength of soil solution and its effect on charge properties of some New Zealand soils, Eur. J. Soil Sci. 33 (1982) 249–262.

[43] Y. Wang, B. Gao, V.L. Morales, Y. Tian, L. Wu, J. Gao, L. Yang, Transport of titanium dioxide nanoparticles in saturated porous media under various solution chemistry conditions, J. Nanopart. Res. 14 (2012) 1–9.

[44] Y.Zhang, Y. Chen, P. Westerhoff, J. Crittenden, Impact of natural organic matter and divalent cations on the stability of aqueous nanoparticles, Water Res. 43 (2009) 4249–4257.

[45] S.H. Joo, S.R. Al-Abed, T. Luxton, Influence of carboxymethyl cellulose for the transport of titanium dioxide nanoparticles in clean silica and mineral-coated sands, Environ. Sci. Technol. 43 (2009) 4954–4959.

[46] N. Solovitch, J. Labille, J. Rose, P. Chaurand, D. Borschneck, M.R. Wiesner, J.Y. Bottero, Concurrent aggregation and deposition of TiO_2 nanoparticles in a sandy porous media, Environ. Sci. Technol. 44 (2010) 4897–4902.

[47] J. Fang, X.Q. Shan, B. Wen, J.M. Lin, G. Owens, Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns, Environ. Pollut. 157 (2009) 1101–1109.

[48] G.V. Lowry, K.B. Gregory, S.C. Apte, J.R. Lead, Transformations of nanomaterials in the environment, Environ. Sci. Technol. 46 (2012) 6893–6899.

[49] M. Hänsch, C. Emmerling, Effects of silver nanoparticles on the microbiota and enzyme activity in soil, J. Plant. Nutr. Soil. Sci. 173 (2010) 554–558.

[50] B.P. Colman, C.L. Arnaout, S. Anciaux, C.K. Gunsch, M.F. Hochella Jr., B. Kim, G.V. Lowry, B.M. McGill, B.C. Reinsch, C.J. Richardson, J.M. Unrine, J.P. Wright, L. Yin, E.S. Bernhardt, Low concentrations of silver nanoparticles in biosolids cause adverse ecosystem responses under realistic field scenario, PLOS ONE 8 (2013) e57189.

[51] M. Debeljak, D. Kocev, W. Towers, M. Jones, B. Griffiths, P. Hallett, Potential of multi-objective models for risk-based mapping of the resilience characteristics of soils, demonstration at a national level, Soil Use Manage. 25 (2009) 66–77.

SUPPLEMENTARY INFORMATION

Table S1 Concentration of the main elements in the 6 soils

Concentrations in mg kg^{-1}	Ag	Al	B	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Sr	Zn
Sandy-Loam (Low OM)	0.46	10888	16.01	1195	1.80	6.05	32.53	17.06	11867	6430.3	143.73	290.43	1445.9	15.55	33.26	18.85	47.52
Sandy-Loam (High OM)	0.45	10357	15.84	1189	1.25	5.43	27.80	21.70	8352	7668.7	151.58	274.41	1423.4	11.00	32.21	18.32	50.66
Loam (Low OM)	0.40	12011	27.13	1868	1.25	5.82	37.65	10.71	10159	6762.8	867.25	545.35	2203.6	13.48	38.60	42.27	47.24
Loam (High OM)	0.66	5509	65.95	3499	1.65	8.67	57.93	26.51	12154	7771.2	185.28	671.21	2557.5	24.93	48.22	34.25	84.11
Silty-Clay (Low OM)	0.77	4835	83.81	825.8	2.49	18.50	83.38	18.78	18108	8920.1	51.44	768.08	1605.5	37.89	32.40	18.96	112.28
Silty-Clay (High OM)	0.69	9314	74.62	3026	2.22	15.18	66.88	20.49	16064	8725.3	570.44	618.65	1333.7	37.57	31.68	17.89	117.00

Table S2 Relationship between C-mineralization and TiO_2-NPs and soil variables tested by multiple regression to identify the main drivers of C-mineralization in soils and to determine which soil physicochemical properties influence TiO_2-NPs toxicity. The following variables were included in the model: TiO_2-NPs treatments, cation exchange capacity (CEC), ionic strength, soil pH, OM content and clay content. To determine if TiO_2-NPs toxicity was related to soil physicochemical properties, the interactions between TiO_2-NPs and the different soil variables were tested. Analysis was performed on the complete set of data, including the 6 soils and the 3 sampling times (n=324). Values in bold are significant.

Variables	P-value	
TiO_2	**0.02**	*
CEC	**< 0.001**	***
Ionic Strength	**< 0.001**	***
pH	**< 0.001**	***
OM content	**< 0.001**	***
Clay content	**< 0.001**	***
TiO_2 x CEC	0.56	
TiO_2 x Ionic Strength	0.46	
TiO_2 x pH	**0.03**	*
TiO_2 x OM content	**0.04**	*
TiO_2 x Clay content	0.58	
Multiple regression model	***P*-value** < 0.001	***
Model R^2	0.87	

4. Conclusions du chapitre

Dans ce chapitre, le but des expérimentations était de déterminer les propriétés clés du sol qui conditionnent le devenir et la toxicité des NPs d'oxydes métalliques. Nous avons pu mettre en évidence que quel que soit le sol considéré, les NPs de TiO_2 et de CuO sont très peu mobiles et que leur transport pouvait être affecté par la matière organique dissoute présente dans la solution du sol, en accord avec divers travaux menés dans des milieux simplifiés. Cependant, la teneur en argile n'apparait pas comme un paramètre clé influençant le transport des NPs étudiées.

La faible mobilité observée des NPs suggère qu'elles sont rapidement filtrées et retenues dans les premiers centimètres du sol. Le risque de transport de ces contaminants hors du site de pollution initial semble donc faible. Ces résultats encouragent à évaluer quelle est la toxicité de ces composés à la suite de leur introduction dans les sols sur le long terme.

De manière générale, nos résultats indiquent que les TiO_2-NPs ont un faible potentiel toxique vis-à-vis de la communauté microbienne hétérotrophe dans les sols étudiés et ce, quelle que soit la concentration appliquée. Dans le sol limono-argileux où une diminution significative de la minéralisation du carbone a été observée, nous n'avons pas observé d'effet dose-réponse contrairement à ce qui était attendu. L'utilisation de six sols aux propriétés contrastées nous a permis d'identifier le pH et la MO comme des paramètres potentiellement impliqués dans la toxicité des NPs. Ceci nécessiterait cependant d'être approfondi en s'intéressant plus particulièrement au rôle de la nature et de la concentration en carbone organique dissout. Par ailleurs, la généralisation de nos conclusions passe par la confirmation des observations à partir d'un panel plus large de sols présentant des propriétés physico-chimiques contrastées.

Dans cette partie du travail, la toxicité des NPs a été abordée en considérant les effets au niveau d'un processus microbien global reposant sur une communauté diversifiée présentant une forte redondance fonctionnelle. Dans la mesure où des processus clés du fonctionnement du sol sont assurés par des groupes fonctionnels moins diversifiés et présentant une redondance moins importante (cf. chapitre 1 § 6-d), nous avons choisi d'évaluer également les effets des NPs en ciblant les communautés nitrifiante et dénitrifiante.

Par ailleurs, l'absence de relation dose-réponse observée étant surprenante, nous avons entrepris de vérifier l'absence de relation linéaire entre la concentration appliquée et la toxicité observée des TiO_2-NPs dans les sols en utilisant une gamme de concentrations en TiO_2 plus large. Le sol limoneux-argileux étant le seul dans lequel un effet toxique des NPs avait été observé, il a été retenu comme sol modèle pour la suite des investigations de ce travail.

Chapitre 3 : Impact du TiO_2 sur le cycle de l'azote : exemple d'un sol limoneux-argileux

1. Introduction

Dans le chapitre 2, nous avons montré que le cycle du carbone pouvait être affecté par les TiO_2-NPs dans un sol limono-argileux. Les processus microbiens du cycle de l'azote sont également cruciaux pour le fonctionnement biologique des sols. C'est pourquoi nous avons évalué les conséquences de la présence de ce polluant émergent sur les processus microbiens de nitrification et dénitrification. La nitrification est un processus souvent étudié en écotoxicologie car il est très sensible à la présence de polluants et est donc un indicateur sensible de perturbation des sols et de leur qualité.

Dans ce chapitre, nous nous sommes intéressés à l'impact de contaminations aigüe (Article 4 et 5) et chronique (Article 6) aux TiO_2-NPs sur la nitrification et la dénitrification dans un sol limono-argileux à fort taux de MO. Les effets ont été évalués sur les activités, les abondances et la diversité des groupes fonctionnels qui sont des indicateurs complémentaires pour décrypter l'impact d'une perturbation sur ces processus microbiens. Une approche originale en écotoxicologie par path analysis, permettant d'étudier les relations causales entre les différentes variables mesurées, a été utilisée en complément afin de comprendre comment la nitrification et la dénitrification étaient affectées par ce type de contaminant.

2. Effet d'une contamination aigüe sur le cycle de l'azote d'un sol

a. Article 4 : Présentation générale de l'étude et synthèse des principaux résultats

Dans le chapitre précédent, nous avons constaté que la minéralisation du carbone du sol limono-argileux (forte MO) était réduite d'environ 20 % quelle que soit la concentration de TiO_2-NPs. Dans la même expérimentation en microcosmes, nous avons donc aussi évalué les effets du TiO_2 sur la nitrification et la dénitrification. La nitrification est considérée comme étant un des processus microbiens les plus sensibles aux polluants alors que la dénitrification reste généralement peu affectée par ce type de perturbation (Bisset *et al.,* 2013). Toutefois, aucune étude n'est encore disponible sur l'évaluation de l'impact des TiO_2-NPs sur ces 2 étapes clés du cycle de l'N dans les sols.

Dans cette partie, nous avons évalué les conséquences des TiO_2-NPs sur les activités nitrifiantes et dénitrifiantes potentielles (NEA et DEA), ainsi que sur l'abondance des groupes fonctionnels impliqués (AOA, AOB et dénitrifiants portant les gènes *nirK* et *nirS*) par PCR quantitative (qPCR). Nous avons également étudié par séquençage haut-débit MiSeq (Illumina), l'impact du TiO_2 sur la diversité des communautés bactériennes et archées totales et impliquées dans l'oxydation de l'ammonium (AOB et AOA).

Nous avons observé que les activités nitrifiantes et dénitrifiantes ont été réduites en présence des 2 concentrations de TiO_2-NPs testées. La nitrification a été particulièrement affectée après 90 jours avec une diminution de 40%, tout comme les abondances des AOA et des AOB qui ont perdu 60 et 40%, respectivement, aux deux concentrations testées, sauf pour les AOB qui ne sont impactées qu'à 500 mg de NPs kg^{-1} de sol. Aucun effet n'a été observé sur l'abondance des dénitrifiants. La diversité des archées, évaluée en ciblant les gènes *16S ADNr* et *amoA,* s'est révélée très faible avec la dominance très marquée d'un OTU (Operational Taxonomic Unit) du genre *Nitrososphaera* qui est une AOA appartenant au phylum des *Thaumarcheota*. L'abondance de cet OTU a été fortement réduite par les 2 doses de TiO_2-NPs et était bien corrélée à la nitrification et à l'abondance des AOA. L'évaluation de l'ensemble de ces résultats par path analysis suggère que les effets négatifs du TiO_2 sur l'OTU dominant *Nitrososphaera* expliquent en grande partie la diminution de l'activité nitrifiante observée qui a ensuite entrainé une diminution en cascade de l'activité dénitrifiante.

Nos résultats indiquent donc que dans le sol étudié la nitrification est un processus sensible aux TiO_2-NPs qui repose en grande partie sur l'activité des AOA *Nitrososphaera.* Ces dernières présentent une très faible diversité et donc une faible redondance fonctionnelle, ce qui a pour conséquence une faible résistance vis-à-vis des NPs. Comme attendu, la dénitrification a été moins affectée. Cependant, le fort couplage entre nitrification et dénitrification a conduit à une diminution de cette activité même si l'abondance des dénitrifiants est restée inchangée.

b. « Response of soil microbial community to titanium dioxide nanoparticles: a cascading pitch on the nitrogen cycle »

L'article 4 intitulé «Response of soil microbial community to titanium dioxide nanoparticles: a cascading pitch on nitrogen cycle» est en préparation pour une soumission dans *The ISME Journal*.

Response of soil microbial community to titanium dioxide nanoparticles: a cascading pitch on the nitrogen cycle

Marie SIMONIN[1,2], Agnès RICHAUME[1], Julien GUYONNET[1], Audrey DUBOST[1], Jean M. F. MARTINS[2] and Thomas POMMIER[1]

[1] UMR CNRS 5557, Laboratoire d'Ecologie Microbienne, Université Lyon1, Université de Lyon, USC INRA 1364, bât G. Mendel, 43 boulevard du 11 novembre 1918, F-69622 Villeurbanne Cedex, France.

[2] LTHE, UMR 5564 CNRS – Univ. Grenoble Alpes 38041 Grenoble Cedex 9, France

Running title: Effects of TiO_2 nanoparticles on the N cycle

ABSTRACT

Soils are facing new environmental stressors, such as titanium dioxide nanoparticles (TiO_2-NPs). While these emerging pollutants are increasingly released into most ecosystems, including agricultural fields, their potential impact on soil and its functioning remain to be investigated. Here we report the response of the microbial community of an agricultural soil (silty-clay texture) exposed over 90 days to TiO_2-NPs at 1 and 500 mg kg^{-1} dry soil. To assess their impact on soil functioning, we focused on the nitrogen cycle and thus measured nitrification and denitrification enzymatic activities combined with the quantification of specific representative genes (*amoA* for ammonia-oxidizers, *nirK* and *nirS* for denitrifiers). In addition, the bacterial, archaeal and ammonia-oxidizers diversity changes were investigated. With strong negative impacts on nitrification enzymatic activities and on the abundances of ammonia-oxidizing microbes, TiO_2-NPs triggered a cascading negative effect on denitrification activity and a deep modification of the bacterial community structure after 90 days of exposure even at the lowest and realistic NPs concentrations. These results appeal further research to assess how these emerging pollutants modify the soil ecosystem functioning and the related soil fertility.

INTRODUCTION

Titanium dioxide nanoparticles (TiO_2-NPs) are widely used into day life products such as sunscreens and toothpastes, industrial products like paints, lacquers and papers, as well as during photocatalytic processes such as water treatment (Keller *et al.*, 2013; Mitrano *et al.*, 2015). Consequently, TiO_2-NPs are chronically released into the environment either directly as nanofertilizers or nanopesticides (Servin *et al.*, 2015) or indirectly in agricultural soils through sewage-sludge application as fertilizers (Brar *et al.*, 2010). Despite their recognized importance in soil ecosystem functionning, the current literature lacks thorough investigations of the effect of TiO_2-NPs on soil microbial communities (Simonin and Richaume, 2015).

In soils, microbial communities play key roles in plant productivity, climate regulation or in biogeochemical processes (Falkowski *et al.*, 2008; Schimel and Schaeffer, 2012; Vacheron *et al.*, 2013), such as the nitrogen (N) cycle in which nitrification and denitrification processes control soil inorganic N availability and subsequent soil fertility (Philippot *et al.*, 2013). The strong coupling between these two functions makes the N cycle a good model to study direct and indirect impacts of environmental disturbances. Nitrification is a two-step process oxidizing ammonia into nitrate. It is assume that the rate-limiting in nitrification is the oxidation of ammonia into nitrite (Kowalchuk and Stephen, 2001), performed by both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Prosser and Nicol, 2012). Nitrification is carried out by a group of microorganisms exhibiting a low functional diversity and is one of the most sensitive soil microbial processes to perturbations, such as pollutants contamination (Dalzell *et al.*, 2002; Broos *et al.*, 2005). However, the influence of environmental stressors on AOA is poorly documented (Mertens *et al.*, 2009; Ollivier *et al.*, 2012) because of the recent discovery of their involvement in soil nitrification (Leininger *et al.*, 2006).

Nitrification is followed by denitrification, which is the sequential reduction of oxidized N compounds (NO_3^-, NO_2^-) into gaseous products (NO, N_2O and N_2) (Zumft, 1997). Contrarily to nitrifier community, the ability to perform denitrification is widespread among several phylogenetic groups and this functional guild has been shown to be fairly insensitive to many

toxicants (Bissett *et al.*, 2013). However, the sensitivity of denitrifiers to TiO_2-NPs has not been investigated yet. Moreover, despite a potential resistance of the denitrifiers to this pollutant, a failure of nitrification may still result in a decrease of denitrification.

High concentrations of TiO_2-NPs have been showed previously to partly reduce general processes performed by many members of the microbial community such as microbial respiration and soil enzyme activities (Du *et al.*, 2011; Ge *et al.*, 2011; Simonin *et al.*, 2015). However, the impact of TiO_2-NPs at environmentally relevant concentrations on key processes performed by functional communities with different levels of functional redundancy, such as those involved in the N cycle, has never been investigated to date.

Here we report the effects of TiO_2-NPs on the N cycle in an agricultural soil exposed over 90 days to two concentrations of TiO_2-NPs simulating environmentally realistic contamination (1 mg kg^{-1} dry soil) or an accidental pollution (500 mg kg^{-1} dry soil) (Sun *et al.*, 2014). Microbial activities and abundances were assessed by measuring nitrification and denitrification enzymatic activities (NEA and DEA, respectively) combined to the quantification of specific representative genes (*amo*A for ammonia-oxidizers and *nirK* and *nirS* for denitrifiers). In addition, the effects of TiO_2-NPs on the diversity of *16S rDNA* bacterial and archaeal genes and *amoA* AOA and AOB genes were determined by high throughput sequencing. The consequences of TiO_2-NPs on the N cycle were discussed in the light of the information provided by a path analysis integrating different variables measured in the experiment.

MATERIAL AND METHODS

Nanoparticles, soil and experimental design

The TiO_2-NPs were provided by Sigma Aldrich (St Louis, USA) with a particle size of 21 nm in powder and ≥ 99.5 % purity. The size and surface charge were previously characterized by Dynamic Light Scattering (DLS) (Simonin *et al.*, 2015). The average size and zeta potential of TiO_2-NPs in ultrapure water used to spike the soil were 160 nm ± 7.2 and - 13.4 mV ± 0.5, respectively.

The soil was sampled from the upper 20 cm layer of a permanent pasture at Commarin (Côte d'Or, France), sieved (2 mm) and stored at 4°C before use. It is a silty-clay soil containing 39.1 % clay, 50.8 % loam and 10.1 % sand. The organic matter content was 7.87 %, the CEC 20.1 $cmol^{+}\ kg^{-1}$ and the water holding capacity 51 %. The soil pH (7.7) and the ionic strength (1.37 mM) were measured following ISO 10390 and ISO 11265 procedures, respectively**.** Soil pH was not modified by the addition of TiO_2-NPs throughout the experiment.

The soil was exposed to either a dose of 1 mg or 500 mg kg^{-1} dry soil of TiO_2-NPs suspended in ultrapure water and dispersed in an ultrasonic bath (Bioblock Scientific) for 5 minutes just before use. TiO_2-NPs suspensions were added homogeneously using a multichannel pipette in order to achieve 100 % of the water holding capacity. The soil was thoroughly mixed manually for 10 minutes to ensure a homogeneous distribution of NPs. Control soils received only ultrapure water. Fifty grams (equivalent dry weight) of contaminated soils were placed into microcosms (150 mL plasma flask) sealed with rubber stoppers. Soil microcosms were incubated for 7, 30 or 90 days at 28°C in the dark and were weekly aerated to ensure a renewal of the atmosphere in the flasks. The experimental design resulted in 54 microcosms: 3 concentrations of TiO_2-NPs (0, 1, 500 mg kg^{-1} dry soil) x 3 exposition times x 6 replicates per treatment. At the beginning of the experiment, 6 additional microcosms were prepared to assess the baseline conditions of the soil. For each incubation time, soil subsamples were immediately used for NEA and DEA measurements, and 3 g were stored at -20°C until DNA extraction.

Nitrification Enzymatic Activity (NEA)

NEA was determined following the protocol described in Dassonville *et al.* (2011). Briefly subsamples of fresh soil (3 g dry soil) were incubated with 6 ml of a solution of $(NH_4)_2SO_4$ (50 µg $N\text{-}NH_4^{+}\ g^{-1}$ dry soil). Distilled water was adjusted in each sample to achieve 24 ml of total liquid volume in flasks. The flasks were sealed with Parafilm® and incubated at 28°C under constant agitation (180 rpm). 1.5 ml of soil slurry were regularly sampled after 2h, 4h, 6h, 8h and 10h of incubation, filtered on 0.2 µm pore size and stored in vials at -20°C until measurement of NO_3^{-} concentrations using an ionic chromatography (DX120, Dionex, Salt

Lake City, USA) equipped with a 4×250 mm column (IonPac AS9 HC). NEA was expressed as µg $N\text{-}NO_3\text{-}$ h^{-1} g^{-1} dry soil.

Denitrification Enzymatic Activity (DEA)

Potential denitrification was measured according to Patra *et al*. (2005). Briefly distilled water (1 mL) containing KNO_3 (50 µg $N\text{-}NO_3^-$ g^{-1} dry soil), glucose (500 µg C-glucose g^{-1} dry soil) and glutamic acid (500 µg C-glutamic acid g^{-1} dry soil) were added to fresh soil (10 g equivalent dry soil) placed in a 150 ml plasma flask. The atmosphere was replaced by 90% helium to ensure anaerobic conditions and 10 % C_2H_2 was added to inhibit N_2O reductase activity. The flasks were sealed with rubber stoppers and incubated at 28°C for 6 h. After 2h of incubation, gas samples were analyzed every hour for the remaining incubation (5 hours) to measure N_2O concentration using a gas chromatograph (Micro GC R3000, SRA Instrument, Marcy L'Etoile, France). DEA was expressed as µg $N\text{-}N_2O$ h^{-1} g^{-1} dry soil.

DNA extraction and quantification

DNA was extracted from 0.5 g of frozen soil using the Power Soil™ DNA Isolation Kit (MO BIO laboratories, Carlsbad, CA, USA) following the manufacturer's instructions and then quantified using the Quant it™ Picogreen© dsDNA Assay kit (Molecular Probes, USA). Fluorescence was measured using a UV spectrophotometer Xenius (Safas, Monaco) (λ=520 nm).

Abundance of nitrifying bacteria (AOA and AOB)

The abundance of AOA and AOB was measured by quantitative PCR targeting *amoA* gene sequences encoding for an ammonia monooxygenase specific for each group. Amplification was performed using gene primers amoA_1F and amoA_2R for the AOB (Rotthauwe *et al.*, 1997) and for the AOA (Tourna *et al.*, 2008). The final reaction volume was 20 µL and contained (final concentrations) 0.5 µM of each primer for the bacterial *amoA* or 0.75µM of CrenamoA616r and 1 µM of CrenamoA23f for the archaeal *amoA*, 2 % bovine serum albumin

(BSA), 1X of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 10 ng of soil DNA extract or of DNA standards with 10^2 to 10^7 gene copies / µl using a constructed linearized plasmid containing archaeal (54d9 fosmide fragment, (Treusch *et al.*, 2005)) and bacterial (*Nitrosomonas europaea*, GenBank accession number:L08050) *amoA* genes. The samples were run in duplicate on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: for bacterial *amoA* 15 min at 95°C, 45 amplification cycles (30 s at 95°C, 45 s at 54°C, 45 s at 72°C and 15 s at 80°C) and 30 s at 40°C ; for archaeal *amoA*, 15 min at 95°C, 50 amplification cycles (45 s at 94°C, 45 s at 55°C and 45 s at 72°C) and 10 s at 40°C. Melting curves analysis confirmed the amplification specificity.

Denitrifying bacteria abundances

The abundance of denitrifying bacteria was measured by quantitative PCR of the genes *nirK* and *nirS* respectively encoding copper- and cytochrome cd1-containing nitrite reductase. Amplification was performed using nirK876 and nirK1040 gene primers (Henry *et al.*, 2004) or nirSCd3aF and nirSR3cd (Kandeler *et al.*, 2006). The final reaction volume for *nirK* quantification was 20 µL and contained (final concentrations) 1 µM of each primer, 0.02 µg of T4 gene protein 32 (QBiogene, Illkirch, France), 1X of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 5 ng of soil DNA extract or of standards with 10^2 to 10^7 copies of DNA (*Sinorhizobium meliloti* 1021). The samples were run in duplicate on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: 15 min at 95°C, 45 amplification cycles (15 s at 95°C, 30 s at 63°C and 30 s at 72°C) and 10 s at 40°C. The final reaction volume for *nirS* quantification was 25 µL and contained (final concentrations) 0.5 µM of each primer, 0.02 µg of T4 gene protein 32 (QBiogene, Illkirch, France), 1X of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 12.5 ng of soil DNA extract or 10^2- 10^7 copies of standard DNA (*Pseudomonas stutzeri* ATCC 14405). The samples were run in duplicate as follows: 15 min at 95°C, 40 amplification cycles (15 s at 95°C, 30 s at 66°C, 30 s at 72°C and 15 s at 80°C) and 10 s at 40°C. Melting curves analysis confirmed the amplification specificity.

Diversity of bacteria, archaea and ammonia-oxidizers

High-throughput sequencing of bacterial and archaeal *16S rDNA* genes and *amoA* AOB and AOA genes (Table 1 and Table S1) were performed on an Illumina MiSeq® platform by Molecular Research DNA, USA. Sequencing was performed on the samples obtained after 7 or 90 days of incubation. A combination of the tools available from the RDP FunGene website (Fish *et al.*, 2013) and the open-source software Mothur (v.1.33.3) (Schloss *et al.*, 2009) was used to process and analyze the sequence data (Table 1). Sequencing products were first paired in overlapping pair-ends, except for *amoA* (AOA) amplicons that were too long (> 500 bp) for the MiSeq technology. Resulting sequence data were then sorted according to their length, and the quality of the primers (< 2 errors) and barcodes (< 1 error). The primers and barcodes were trimmed off before searching potential chimeric formation using UCHIME (Edgar *et al.*, 2011) implemented in Mothur. Putative chimeras were removed from the dataset. For *amoA* sequences, nucleotide sequences were translated in amino-acids and possible frame-reading shifts were detected and corrected using the FRAMEBOT algorithm (Wang *et al.*, 2013).

We clustered the corrected *16S* sequences into operational taxonomic units (OTUs) by setting a 0.03 distance limit (Kim *et al.*, 2011). The *amoA* sequences were clustered at 0.05 dissimilarity. The taxonomic identification of bacterial and archaeal *16S rDNA* genes was performed using the Greengenes 13.5 database. Similarity of *amoA* sequences with known references were assessed with 7 652 sequences of *amoA* AOB and 9 326 sequences of *amoA* AOA extracted from the FunGene database. The relatedness of *amoA* AOA sequences with identified archaeal taxa was performed using the database assembled by Pester *et al*. (2012). Rarefaction curves based on identified OTU and species richness estimator ACE were generated using Mothur for each sample. Singleton reads were not considered for subsequent analyses. Non-metric Multi-Dimensional Scaling (NMDS) were performed using the *metaMDS* function, available in the Vegan package (Oksanen *et al.*, 2007) of the R software (R Core Team, 2015) based on Bray-Curtis distances, associated to Permanova (Permutational multivariate analysis of variance) using the *adonis* function, to explore the difference in community composition in the different treatments.

Table 1 Main information on the diversity analysis of bacterial, archaeal, AOB and AOA communities

Community (Gene)	Primers	Mean Fragment size	Mean sequence number per sample	Total sequence number analyzed
Bacteria (*16S rDNA*)	515F / 806R (V4 Region)	300 bp	4580 ± 166	160301
Archaea (*16S rDNA*)	349F / 806R (V3-V4 Region)	424 bp	5745 ± 3332	206831
AOB (*amoA*)	amoA_1F / amoA_2R	435 bp	11745 ± 451	422844
AOA (*amoA*)	CrenamoA23F / CrenamoA616R	252 bp	16929 ± 9545	609456

Statistical analysis

All results are presented as means (± standard errors). A one-way analysis of variance (ANOVA) and *post-hoc* Tukey HSD were performed to test the effect of TiO_2-NP concentrations on measured variables at each sampling time. Where necessary, data were log-transformed prior to analysis to ensure conformity with the assumptions of normality and homogeneity of variances. Linear regressions were conducted to investigate relationships between all variables measured in the experiment, described by Spearman correlation coefficient (*r)*. Effects with $P < 0.05$ are referred to as significant. All statistical analysis was carried out using the R statistical software 2.13.2 (R Core Team, 2011).

Path analysis (Shipley, 2002) was performed using Amos18® (Amos Development Corporation, Crawfordville, FL, USA) to explore the causal links between TiO_2 concentration, nitrifier abundance, denitrifier abundance, nitrification and denitrification activities. χ^2 test was used to evaluate model fit, by determining whether the covariance structures implied by the model adequately fit the actual covariance structures of the data. A non-significant chi-squared test ($P > 0.05$) indicates adequate model fits. The coefficients of each path as the calculated standardized coefficients were determined using the analysis of correlation matrices. These coefficients indicate by how many standard deviations the effect variable would change if the causal variable was changed by one standard deviation. The indirect effects of a variable can be calculated by the product of the coefficients along the path. Paths in this model were considered significant with a *P*-value < 0.05.

RESULTS

Impact of TiO_2-NPs on soil functioning

NEA and DEA were considered as relevant proxies of soil functioning. Both TiO_2-NPs concentrations reduced NEA compared to the control after 30 days with 31% decrease (P < 0.001) and 20% (P = 0.02) for NPs concentrations of 1 and 500 $mg.kg^{-1}$, respectively (Figure 1A). These reductions were stronger after 90 days of incubation with about 40% decrease (P < 0.001) for both concentrations.

DEA was significantly decreased (Figure 1B) at the 3 sampling dates at 500 mg kg^{-1} (7 days: - 15%, P = 0.01; 30 days: - 21%, P = 0.04; 90 days: - 19%, P < 0.001). At 1 mg kg^{-1}, a significant reduction of DEA was only observed after 90 days of incubation (- 15%, P < 0.001) in comparison with the control.

Impact of TiO_2-NPs on N-related functional guild abundances

Overall, the abundance of *amoA* AOA gene was 75 fold higher than the *amoA* AOB abundances in the studied soil, with an average of 9.27 x 10^7 and 1.25 x 10^6 copies for AOA and AOB, respectively. The dynamics of *amoA* AOA and *amoA* AOB under TiO_2-NP treatments were contrasted (Figure 2). While the application of 500 mg kg^{-1} TiO_2-NPs lead to a decrease of *amoA* AOB gene abundance only after 90 days (Figure 2A, - 39 %, P = 0.05), no variation was observed at 1 mg kg^{-1} during the course of the experiment. At the opposite, the abundance of *amoA* AOA was strongly decreased whatever the concentration after 90 days (Figure 2B, dose 1: - 53 %, P < 0.001; dose 500: - 60 %, P < 0.001). NEA was negatively correlated with the abundance of *amoA* AOB (R^2 = 0.18, P = 0.002, Table 2) but was positively correlated with the abundance of *amoA* AOA (R^2 = 0.26, P < 0.001, Table 2).

The abundance of *nir*S gene was 4.5 fold higher than the abundance of *nirK*, with an average of 1.15 x 10^7 and 2.57 x 10^6 copies per gram of dry soil, respectively. The *nirK* and *nirS* genes abundances were not significantly modified in the microcosms exposed to TiO_2-NPs over the course of the experiment (Figure 2C and 2D). DEA was not correlated to the abundance of *nirS* but was positively correlated with the abundance of *nirK* (R^2 = 0.28, P < 0.001, Table 2).

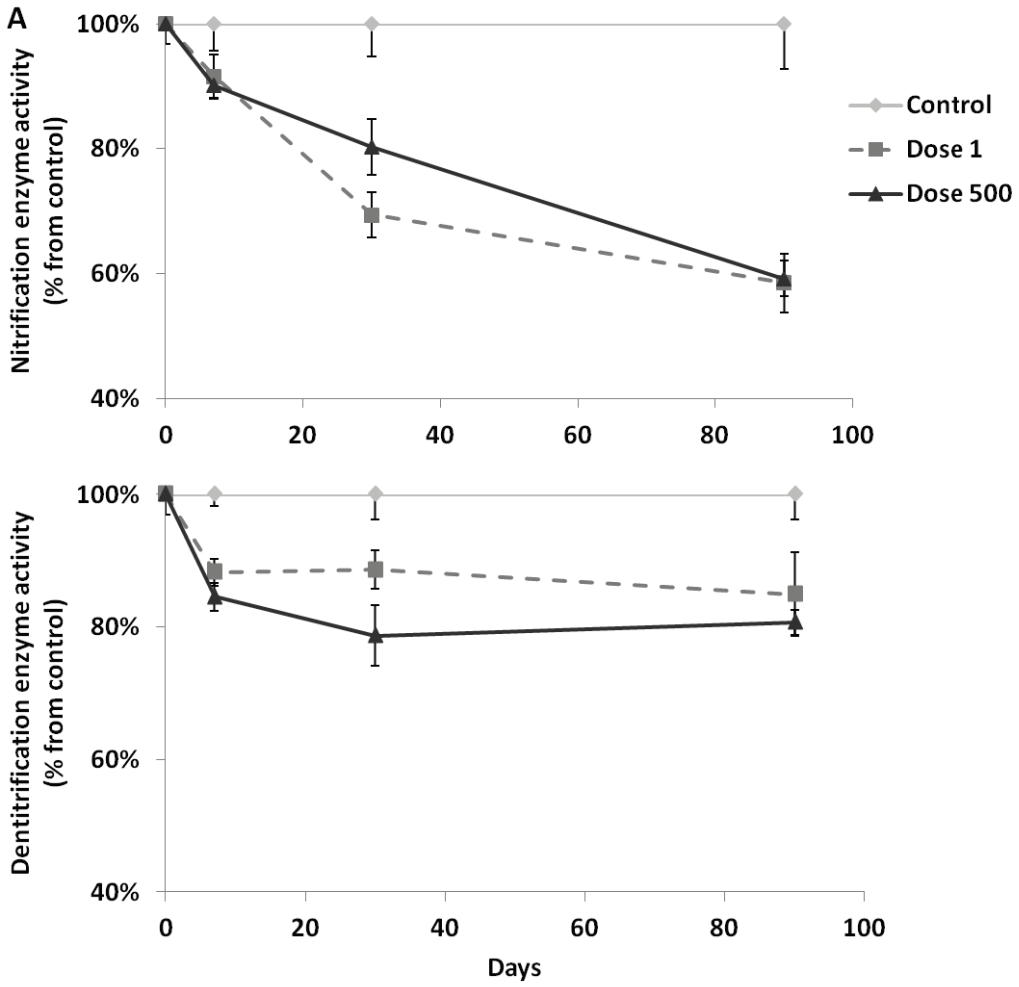


Figure 1 Changes over time of NEA (A) and DEA (B) in the different treatments (grey line: Control, dotted line: Dose 1 mg kg^{-1}, black line: Dose 500 mg kg^{-1} dry soil). Error bars represent the standard error (n=6).

Table 2 Correlation table between the different measured variables (AOB abundance, AOA abundance, *nirK* abundance, *nirS* abundance, NEA and DEA). The strength of the linear relationship is given by the correlation coefficient *r* and negative numbers correspond to negative correlations, *P*-values are given in parenthesis. Significant correlations are presented in bold. Spearman correlations were investigated on all data of the 3 sampling times (n=54).

	AOB	**AOA**	***nirK***	***nirS***	**NEA**
AOB					
AOA	0.06 (0.69)				
nirK	0.04 (0.79)	-0.02 (0.94)			
nirS	0.22 (0.11)	**0.27 (0.03)**	-0.17 (0.20)		
NEA	**-0.43 (0.002)**	**0.51 (<0.001)**	**0.35 (0.02)**	0.01 (0.97)	
DEA	**-0.39 (0.004)**	0.20 (0.10)	**0.53 (<0.001)**	-0.08 (0.69)	**0.68 (<0.001)**

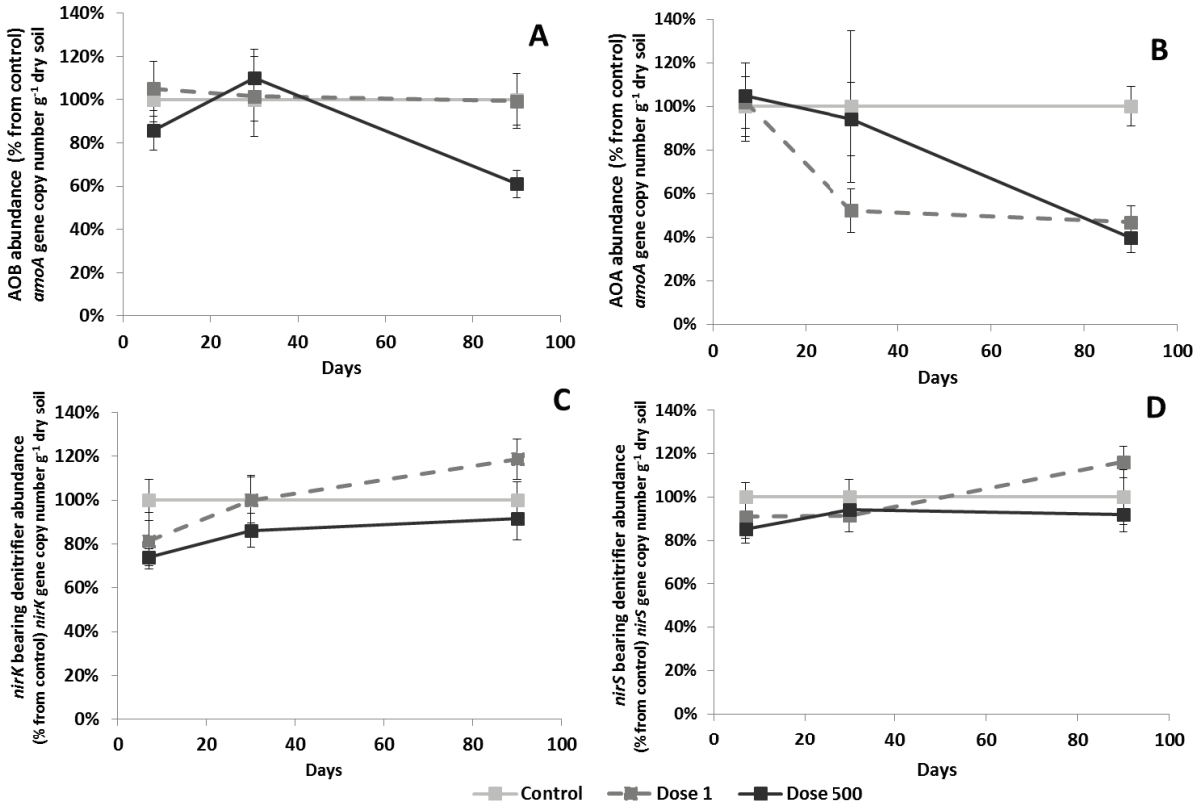


Figure 2 Changes over time of (A) ammonia-oxidizing bacteria (AOB), (B) ammonia-oxidizing archaea (AOA), (C) *nirK* bearing denitrifier abundance and (D) *nirS* bearing denitrifier abundance in the different treatments (grey line: Control, dotted line: Dose 1 mg kg^{-1}, black line: Dose 500 mg kg^{-1} dry soil). Error bars represent the standard error (n=6).

Impact of TiO_2-NPs on soil microbial diversity

We assessed the impact of TiO_2-NPs on soil microbial diversity by sequencing the *16S rDNA* and *amoA* bacterial and archaeal genes.

160 301 original *16S rDNA* bacterial sequences yielded a total of 8 256 OTUs (average per sample: 1 466 ± 8.85 OTUs) at a distance dissimilarity of 0.03. No significant change in richness (ACE estimator) was observed for this gene during the experiment and between treatments. However TiO_2-NPs contaminations, time and the interaction of time and exposure caused a significant shift in the bacterial community structure (TiO_2-NPs: $P < 0.001$, time: $P < 0.001$, TiO_2-NPs x time: $P = 0.005$, Figure 3A). Both doses of TiO_2-NPs significantly affected the bacterial community structure (Dose 1: $P = 0.001$, Dose 500: $P = 0.003$) after 90 days and the relative abundance of several OTUs were either lower (e.g. *Acidobacteria* group 6, *Saprospirae*) or higher (e.g. *Sulfuritalea*, *Anaerolinea*, *Bacteroidia*) than the control (Figure S1). After 90 days, the shift in the bacterial community structure induced by TiO_2-NP contamination was associated to a modification of the relative abundance of different phyla, especially the *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Chloroflexi* phyla (Figure S2).

The archaeal community diversity assessed by *16S rDNA* gene sequencing exhibited a low richness with a total of 364 OTUs (average per sample: 28.75 ± 0.91 OTUs) from 206 831 original sequences at a dissimilarity distance of 0.03. In all samples, the community was dominated by a single OTU (relative abundance in average per sample: 70.4 % ± 2 %) belonging to an ammonia-oxidizing archeon of the genus *Nitrososphaera* (Figure S3). The archaeal community structure was modified during the incubation (time: $P = 0.002$, Figure 3C), but not in association with any TiO_2-NP exposure ($P = 0.34$). However, the number of sequences belonging to the dominant *Nitrososphaera* was significantly reduced in the microcosms exposed to both concentrations of TiO_2-NPs after 90 days (Dose 1: - 58 %, $P = 0.04$; Dose 500: - 59 %, $P = 0.04$, Figure 4A). Interestingly, the abundance of *amoA* AOA and the NEA were positively correlated with the number of *16S rDNA* gene sequence representing this dominant *Nitrososphaera* OTU (Figure 4C and 4D, respectively). After 90 days under TiO_2-NP exposure, while the number of *16S rDNA* gene sequence of this

dominant *Nitrososphaera* OTU decreased, the number of *16S rDNA* gene sequences of the OTU belonging to the *Methanocella* genus was increased (relative abundance, in control: 0.3 % ± 0.08 %; in TiO_2-NP treatments 12 % ± 3 %, Figure S3).

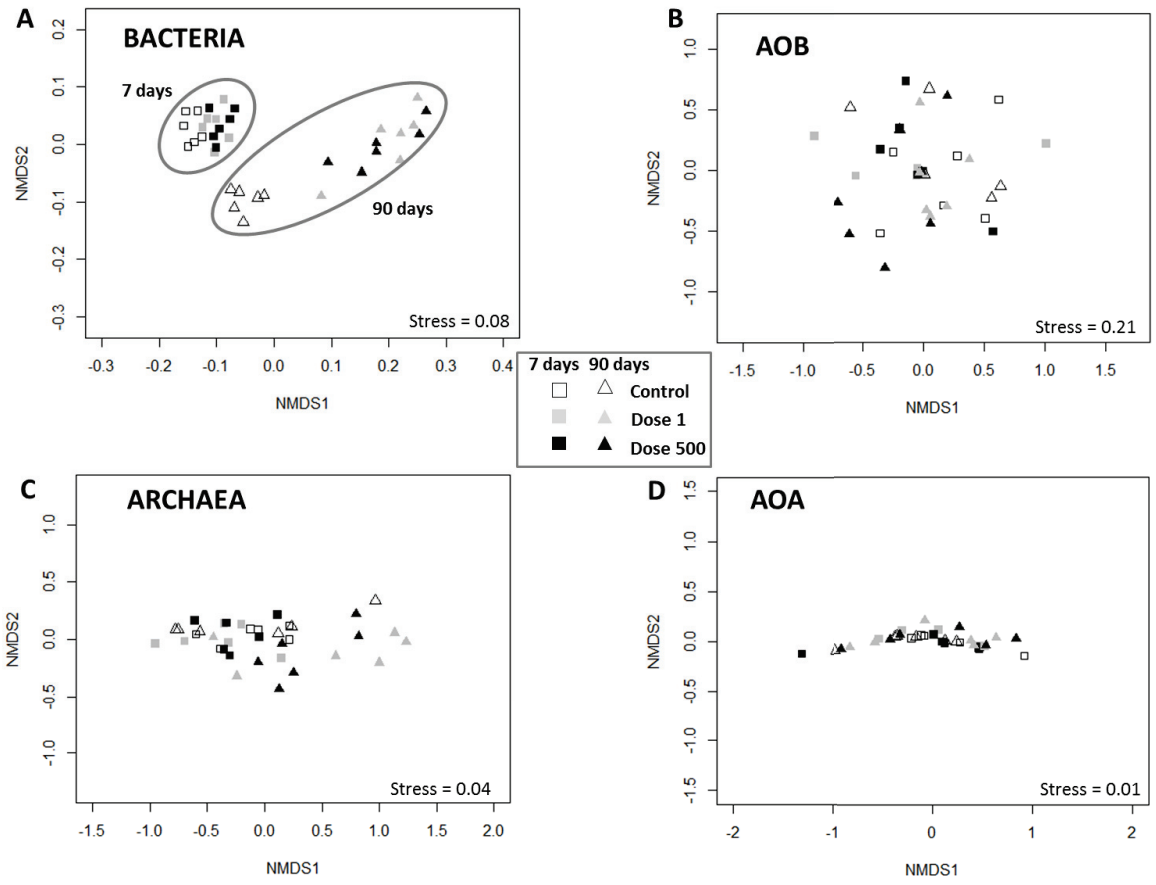


Figure 3 Nonmetric Multidimensional Scaling analysis to determine the modification in microbial community structure in presence of TiO_2-NPs (grey: Control, blue: Dose 1, red: Dose 500) after 7 days (square symbols) and 90 days (triangle symbols): A) Bacterial community, B) AOB community, C) Archaeal community, D) AOA community.

422 844 original *amoA* AOB gene sequences clustered into 5974 total clusters (average per sample: 272 ± 2.5 clusters) at a dissimilarity of 0.05. The ACE richness estimator of the AOB community was significantly decreased after 90 days in presence of 500 mg kg^{-1} of TiO_2-NPs (- 8.4 %, P = 0.004). However the AOB community structure did not vary in the microcosms exposed to TiO_2-NPs throughout the incubation (TiO_2-NPs: P = 0.68, time: P = 0.21, Figure 3B).

609 456 original *amoA* AOA gene sequences yielded 2103 total clusters (average per sample: 44 ± 8 clusters) at a dissimilarity of 0.05. The AOA community structure assessed by *amoA* AOA gene sequences was not modified when exposed to TiO_2-NPs or during the incubation (TiO_2-NPs: P = 0.95, time: P = 0.31, Figure 3D). In all samples, similar to the results of *16S rDNA* sequences, the *amoA* AOA sequences were dominated by a single cluster belonging to *Nitrososphaera* cluster (Figure S3), which represent in average 91 % of all sequences analyzed. Moreover, the other clusters obtained were also affiliated to this cluster (Figure S3). Contrary to the dominant *16S rDNA Nitrososphaera* OTU, the number of sequences belonging to the dominant *amo*A *Nitrososphaera* cluster was not affected by TiO_2-NPs exposure at 7 or 90 days (Figure 4B). Furthermore, this dominant *amoA Nitrososphaera* cluster was not correlated to NEA, AOA abundance or to the dominant 16S *Nitrososphaera* OTU sequence number (data not shown).

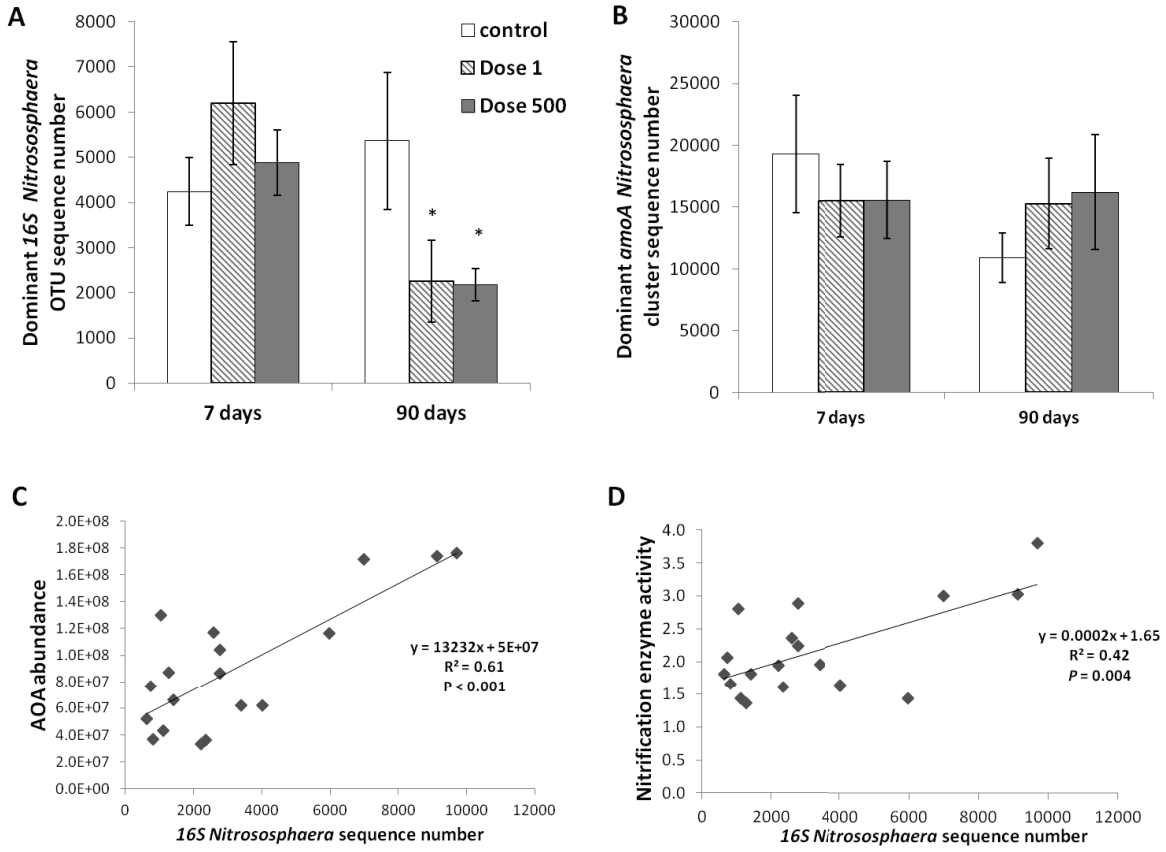


Figure 4 Effects of TiO_2-NPs A) on the dominant archaeal *16S rDNA* OTU affiliated to the *Nitrososphaera* genus in the different treatments, B) on the dominant AOA *amoA* cluster

affiliated to the *Nitrososphaera* cluster (white bars: Control, hashed bars: Dose 1 mg kg^{-1}, black bars: Dose 500 mg kg^{-1} dry soil). C) Correlation between the dominant *16S Nitrososphaera* sequence number and AOA abundance (n=36). D) Correlation between the dominant *16S Nitrososphaera* sequence number and NEA. The regression is drawn and the R^2 and *P*-values are given.

Potential causal relationships between TiO_2 contamination, microbial communities and soil functioning

We investigated potential causal relationships between TiO_2 contamination and microbial activity, abundance and diversity using path analysis. Path analysis was performed from the data obtained after 90 days of exposure to TiO_2-NPs because their effects were the greatest on microbial activities and abundances (Figure 1 and 2). The variables included in the model were significantly correlated to NEA or DEA. The model explained a significant part of NEA (70 %) and DEA (75 %) variances. TiO_2-NPs significantly and negatively affected the abundance of the dominant *16S Nitrososphaera* OTU (path coefficient = - 0.28, *P* = 0.02) and also of *amoA* AOA and AOB genes (path coefficient = - 0.30, *P* = 0.03 and - 0.61, *P* = 0.001 respectively). The number of *16S rDNA* gene sequences of the dominant archaeal *Nitrososphaera* was an important driver of the AOA abundance (path coefficient = 0.70, *P* < 0.001). The path analysis supported the relative abundance of *amoA* AOA genes as the major driver of NEA in this soil (path coefficient = 0.77, *P* < 0.001) and the relative abundance of *amoA* AOB as a minor actor of NEA (path coefficient = 0.17, *P* = 0.23). A great part of the variance of NEA was explained by indirect effects of the dominant *16S Nitrososphaera* OTU (indirect effect = 0.53). TiO_2-NPs influenced DEA through direct effects (path coefficient = - 0.38, *P* = 0.007) and not indirect effects via *nirK* relative abundance (indirect effects = - 0.05). The *nirK* gene abundance was a driver of DEA (path coefficient = 0.28, *P* = 0.03) but DEA was mainly explained by NEA activity (path coefficient = 0.61, *P* < 0.001), suggesting that TiO_2-NP contamination primarily affects this process through a decrease of AOA abundance and NEA activity.

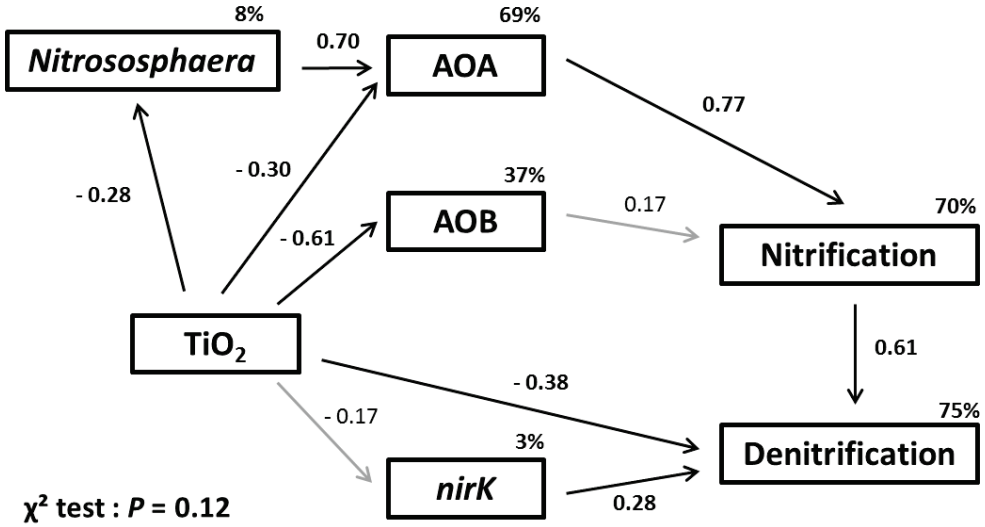


Figure 5 Path analysis of the direct and indirect effects of TiO_2-NPs, dominant *16S rDNA Nitrososphaera* sequence number, AOA abundance, AOB abundance, *nirK* abundance on NEA and DEA. Path coefficients (values indicated next to the arrows) correspond to the standardized coefficients calculated based on the analysis of correlation matrices and indicate by how many standard deviations the effect variable would change if the causal variable was changed by one standard deviation. Arrows and values in bold indicate a significant causal relationship between two variables.

DISCUSSION

Negative effect of a realistic concentration of TiO_2-NPs on the N cycle

TiO_2-NPs caused an important disturbance of the nitrogen cycle and a modification of the bacterial community structure in the agricultural soil studied even for a low realistic concentration (1 mg kg^{-1} dry soil). Moreover, no resilience of the negative effects was observed during the time course of the experiment. Surprisingly, the lowest concentration had similar effects on potential activities and AOA abundance than the highest one (500 mg kg^{-1} dry soil). The absence of linear dose-response of NPs on soil microbial activities has been previously observed (Ge *et al.*, 2011; Jośko *et al.*, 2014; Simonin *et al.*, 2015). A hypothesis could be that the NPs homo- and heteroaggregation processes (i.e. the aggregation of NP with themselves and the aggregation of NP with other environmental constituents) are modified in function of the NPs concentration applied to soil (Lowry *et al.*, 2012) with

consequences on NP bioavailability and toxicity for microorganisms (Cornelis *et al.*, 2014). However, further work focusing on the physicochemical properties of NP in relation with the concentration applied into soil would be needed.

The absence of resilience of the denitrification activity and the increased over time declines of nitrification and ammonia-oxidizers abundance raise concerns about the ecotoxicity of TiO_2-NPs in soil. The greatest effects of TiO_2-NPs appeared 90 days after the contamination suggesting that aged NP can still be toxic to microorganisms even at low concentrations and after a long period of exposure. This should be considered in regards to transport experiments suggesting that TiO_2-NPs exhibit a low mobility in soils and would have a long residence time in these ecosystems (Fang *et al.*, 2009; Nickel *et al.*, 2015). Therefore, our results imply that short term experiments may have not accurately reflected the toxic potential of NP in soil. Most studies are based on short incubation periods not exceeding 60 days with high concentrations of NP (>100 mg kg^{-1}) (Simonin and Richaume, 2015). Considering these results, we strongly encourage further research to use realistic concentrations with longer incubations.

AOA are more affected by TiO_2-NPs than AOB

The AOA abundance was highly reduced by TiO_2-NPs contamination even at low concentration, especially compared to the AOB abundance. In this case, the AOA abundance decreases down to 60% after 90 days of incubation. Consistent with a previous study (Mertens et al. 2009), our results challenge the view that *Archaea* are more tolerant to chronic stresses than *Bacteria* (Schleper *et al.*, 2005; Valentine, 2007). The study of the ecology of AOA in soil and their response to environmental stressors is still at their infancy and very few studies have investigated the impact of pollutants on soil AOA (Schauss *et al.*, 2009; Mertens *et al.*, 2009; Liu *et al.*, 2010; Ollivier *et al.*, 2012, 2013) . To our knowledge, none studied the impacts of NPs. Pure culture studies have shown that TiO_2-NPs can be toxic to *Bacteria* after adsorption to cell membrane causing an oxidative stress associated to reactive oxygen species (ROS) production (Adams *et al.*, 2006; Simon-Deckers *et al.*, 2009). The very limited knowledge of soil *Archaea* physiology and the absence of toxicological

studies using soil archaeal strains do not allow hypothesizing that the same mechanisms are involved in AOA mortality in presence of TiO_2-NPs.

In the studied soil, AOA affiliated to the dominant *Nitrososphaera* genus were likely the main functional drivers of nitrification as suggested by the positive correlations with NEA and an AOA abundance 75-fold higher than AOB abundance. As illustrated by the path analysis, TiO_2-NPs had negative effects on AOA abundance with cascading negative effects on nitrification and denitrification activities. Thus, AOA *Nitrososphaera* had a pivotal role in the response of this soil to TiO_2-NP contamination.

A higher abundance of AOA than AOB in soil exhibiting neutral or alkaline pH have been reported several times (Leininger *et al.*, 2006; Jia and Conrad, 2009). Our results confirmed these observations and pointed out for the first time (to authors' knowledge), that AOA can dominate ammonia-oxidizing activity under alkaline pH and high OM content (Shen *et al.*, 2008; Jia and Conrad, 2009; Xia *et al.*, 2011; Ai *et al.*, 2013; Le Roux *et al.*, 2013).

In our study, the archaeal diversity was very low compared to the bacterial community and the most represented soil archaea (*16S rDNA*) and AOA (*amoA*) was affiliated to *Candidatus Nitrososphaera*. This observation is consistent with the literature reporting that this AOA is dominant in many agricultural soils worldwide (Taketani and Tsai, 2010; Bates *et al.*, 2011; Pester *et al.*, 2012; Zhalnina *et al.*, 2013). Interestingly, this dominant *16S rDNA Nitrososphaera* OTU decreased with TiO_2-NPs exposure and was positively correlated to NEA and AOA abundance. In contrast, the dominant *amoA Nitrososphaera* cluster was not affected by the contamination and was not related to AOA abundance, NEA or *16S rDNA Nitrososphaera* OTU. This result advocates for a lower level of diversity for *amoA* AOA genes than for *16S rDNA* AOA genes (Tourna *et al.,* 2008) and suggests that TiO_2-NPs exposure affected taxonomically different AOA groups harboring similar *amoA* genes. Accordingly for AOB community, we did not observed any particular pattern of *amoA* AOB community structure despite a broad shift in the bacterial community as assessed by *16S rDNA* gene diversity.

Cascading negative effects on denitrification

Unlike the nitrifier community, the abundance of the denitrifying community was not reduced by TiO_2-NPs exposure, yet DEA was constantly decreased (around – 20 %) all along the incubation. The higher resistance and resilience of denitrifiers to pollutants is well acknowledged and can be explained by their high functional redundancy, niche breadth and adaptive ability (Bissett *et al.*, 2013). The path analysis indicated that the decrease of denitrification activity was mainly explained by an indirect effect of the decline of nitrification caused by the decrease of the AOA community, which was related to TiO_2 exposure. However the decline of nitrification did not fully explain the denitrification decrease, because TiO_2-NPs also directly affected denitrification (path coefficient = - 0.38). This path reflects that other variables not included in the model are linked to the decrease of DEA. As suggested by Cantarel et al. (2012), specific clusters of denitrifying bacteria could be better related to denitrification than functional gene abundance (*nirK* and *nosZ*).

Conclusions

Nanomaterials such as TiO_2 are worrying emerging contaminants of terrestrial ecosystem. Their effects not only on soil functioning but also on microbial diversity and abundance of functional guilds are of particular interest for the environmental risk assessment. Our results suggest that direct effects do not explain NPs impact on N cycle. Considering functional links and indirect effects on key actors of this cycle provide an integrative assessment of their impact on the soil microbial community and its functioning. Particularly, soil nitrification and ammonia-oxidation performed by AOA, deserve deeper analyses from both a toxicological and environmental perspectives.

ACKNOWLEDGEMENTS

Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region – ARC Environnement. This work was funded by a grant from the French National Program EC2CO-CNRS AT-Microbien. The authors thank the technical assistance of Morgane Ginot. Microbial measurements were performed at the AME platform (Microbial Ecology UMR5557-USC1364,

Lyon), quantitative PCR at the DTAMB platform (IFR 41, University Lyon 1) and bioinformatic analyses at the Ibio platform (Microbial Ecology UMR5557-USC1364, Lyon).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adams LK, Lyon DY, Alvarez PJJ. (2006). Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. Water Research 40:3527–3532.

Ai C, Liang G, Sun J, Wang X, He P, Zhou W. (2013). Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil. Soil Biology and Biochemistry 57:30–42.

Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N. (2011). Examining the global distribution of dominant archaeal populations in soil. ISME J 5:908–917.

Bissett A, Brown MV, Siciliano SD, Thrall PH. (2013). Microbial community responses to anthropogenically induced environmental change: towards a systems approach. Ecol Lett 16:128–139.

Brar SK, Verma M, Tyagi RD, Surampalli RY. (2010). Engineered nanoparticles in wastewater and wastewater sludge – Evidence and impacts. Waste Management 30:504–520.

Broos K, Mertens J, Smolders E. (2005). Toxicity of heavy metals in soil assessed with various soil microbial and plant growth assays: A comparative study. Environmental Toxicology and Chemistry 24:634–640.

Cantarel AAM, Bloor JMG, Pommier T, Guillaumaud N, Moirot C, Soussana J-F, et al. (2012). Four years of experimental climate change modifies the microbial drivers of N2O fluxes in an upland grassland ecosystem. Glob Change Biol 18:2520–2531.

Cornelis G, Hund-Rinke K, Kuhlbusch T, Brink N van den, Nickel C. (2014). Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. Critical Reviews in Environmental Science and Technology 44:2720–2764.

Dalzell DJB, Alte S, Aspichueta E, de la Sota A, Etxebarria J, Gutierrez M, et al. (2002). A comparison of five rapid direct toxicity assessment methods to determine toxicity of pollutants to activated sludge. Chemosphere 47:535–545.

Dassonville N, Guillaumaud N, Piola F, Meerts P, Poly F. (2011). Niche construction by the invasive Asian knotweeds (species complex Fallopia): impact on activity, abundance and community structure of denitrifiers and nitrifiers. Biol Invasions 13:1115–1133.

Du W, Sun Y, Ji R, Zhu J, Wu J, Guo H. (2011). TiO2 and ZnO nanoparticles negatively affect wheat growth and soil enzyme activities in agricultural soil. J Environ Monit 13:822–828.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. (2011). UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200.

Falkowski PG, Fenchel T, Delong EF. (2008). The Microbial Engines That Drive Earth's Biogeochemical Cycles. Science 320:1034–1039.

Fang J, Shan X, Wen B, Lin J, Owens G. (2009). Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. Environmental Pollution 157:1101–1109.

Fish JA, Chai B, Wang Q, Sun Y, Brown CT, Tiedje JM, et al. (2013). FunGene: the functional gene pipeline and repository. Front Microbiol 4. doi:10.3389/fmicb.2013.00291.

Ge Y, Schimel J, Holden P. (2011). Evidence for negative effects of TiO2 and ZnO nanoparticles on soil bacterial communities. Environmental Science & Technology 45:1659–1664.

Henry S, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A, Philippot L. (2004). Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. Journal of Microbiological Methods 59:327–335.

Jia Z, Conrad R. (2009). Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology 11:1658–1671.

Jośko I, Oleszczuk P, Futa B. (2014). The effect of inorganic nanoparticles (ZnO, Cr2O3, CuO and Ni) and their bulk counterparts on enzyme activities in different soils. Geoderma 232–234:528–537.

Kandeler E, Deiglmayr K, Tscherko D, Bru D, Philippot L. (2006). Abundance of narG, nirS, nirK, and nosZ Genes of Denitrifying Bacteria during Primary Successions of a Glacier Foreland. Appl Environ Microbiol 72:5957–5962.

Keller AA, McFerran S, Lazareva A, Suh S. (2013). Global life cycle releases of engineered nanomaterials. J Nanopart Res 15:1–17.

Kim M, Morrison M, Yu Z. (2011). Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. Journal of Microbiological Methods 84:81–87.

Kowalchuk G, Stephen J. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annual Reviews in Microbiology 55:485–529.

Leininger S, Urich T, Schloter M. (2006). Archaea predominate among ammonia-oxydizing prokaryotes in soils. Nature 442:806–809.

Liu Y-R, Zheng Y-M, Shen J-P, Zhang L-M, He J-Z. (2010). Effects of mercury on the activity and community composition of soil ammonia oxidizers. Environmental Science and Pollution Research 17:1237–1244.

Lowry GV, Gregory KB, Apte SC, Lead JR. (2012). Transformations of Nanomaterials in the Environment. Environ Sci Technol 46:6893–6899.

Mertens J, Broos K, Wakelin SA, Kowalchuk GA, Springael D, Smolders E. (2009). Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. ISME J 3:916–923.

Mitrano DM, Motellier S, Clavaguera S, Nowack B. (2015). Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. Environment International 77:132–147.

Nickel C, Gabsch S, Hellack B, Nogowski A, Babick F, Stintz M, et al. (2015). Mobility of coated and uncoated TiO 2 nanomaterials in soil columns–Applicability of the tests methods of OECD TG 312 and 106 for nanomaterials. Journal of environmental management 157:230–237.

Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MHH, Oksanen MJ, et al. (2007). The vegan package. Community ecology package.

Ollivier J, Schacht D, Kindler R, Groeneweg J, Engel M, Wilke B-M, et al. (2013). Effects of repeated application of sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions. Front Microbiol 4. doi:10.3389/fmicb.2013.00022.

Ollivier J, Wanat N, Austruy A, Hitmi A, Joussein E, Welzl G, et al. (2012). Abundance and Diversity of Ammonia-Oxidizing Prokaryotes in the Root–Rhizosphere Complex of

Miscanthus × giganteus Grown in Heavy Metal-Contaminated Soils. Microb Ecol 64:1038–1046.

Patra A, Abbadie L, Clays-Josserand A, Degrange V, Grayston S, Loiseau P. (2005). Effect of grazing on microbial functional groups involved in soil N dynamics. Ecological Monographs 75:65–80.

Pester M, Rattei T, Flechl S, Gröngröft A, Richter A, Overmann J, et al. (2012). amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions. Environmental Microbiology 14:525–539.

Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology 11:789–799.

Prosser JI, Nicol GW. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends in Microbiology 20:523–531.

R Core Team. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing. http://www.R-project.org/.

Rotthauwe JH, Witzel KP, Liesack W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712.

Le Roux X, Schmid B, Poly F, Barnard RL, Niklaus PA, Guillaumaud N, et al. (2013). Soil Environmental Conditions and Microbial Build-Up Mediate the Effect of Plant Diversity on Soil Nitrifying and Denitrifying Enzyme Activities in Temperate Grasslands. PLoS ONE 8:e61069.

Schauss K, Focks A, Leininger S, Kotzerke A, Heuer H, Thiele-Bruhn S, et al. (2009). Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environmental Microbiology 11:446–456.

Schimel JP, Schaeffer SM. (2012). Microbial control over carbon cycling in soil. Front Microbiol 3. doi:10.3389/fmicb.2012.00348.

Schleper C, Jurgens G, Jonuscheit M. (2005). Genomic studies of uncultivated archaea. Nat Rev Micro 3:479–488.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl Environ Microbiol 75:7537–7541.

Servin A, Elmer W, Mukherjee A, Torre-Roche RD la, Hamdi H, White JC, et al. (2015). A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. J Nanopart Res 17:1–21.

Shen J, Zhang L, Zhu Y, Zhang J, He J. (2008). Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environmental Microbiology 10:1601–1611.

Shipley B. (2002). Cause and Correlation in Biology: A User's Guide to Path Analysis, Structural Equations and Causal Inference. Cambridge University Press.

Simon-Deckers A, Loo S, Mayne-L'hermite M, Herlin-Boime N, Menguy N, Reynaud C, et al. (2009). Size-, Composition- and Shape-Dependent Toxicological Impact of Metal Oxide Nanoparticles and Carbon Nanotubes toward Bacteria. Environ Sci Technol 43:8423–8429.

Simonin M, Guyonnet JP, Martins JMF, Ginot M, Richaume A. (2015). Influence of soil properties on the toxicity of TiO2 nanoparticles on carbon mineralization and bacterial abundance. Journal of Hazardous Materials 283:529–535.

Simonin M, Richaume A. (2015). Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review. Environ Sci Pollut Res 1–14.

Sun TY, Gottschalk F, Hungerbühler K, Nowack B. (2014). Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. Environmental Pollution 185:69–76.

Taketani RG, Tsai SM. (2010). The Influence of Different Land Uses on the Structure of Archaeal Communities in Amazonian Anthrosols Based on 16S rRNA and amoA Genes. Microb Ecol 59:734–743.

Tourna M, Freitag TE, Nicol GW, Prosser JI. (2008). Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology 10:1357–1364.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk H-P, Schleper C. (2005). Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environmental Microbiology 7:1985–1995.

Vacheron J, Desbrosses G, Bouffaud M-L, Touraine B, Moënne-Loccoz Y, Muller D, et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci 4. doi:10.3389/fpls.2013.00356.

Valentine DL. (2007). Adaptations to energy stress dictate the ecology and evolution of the Archaea. Nat Rev Micro 5:316–323.

Wang Q, Quensen JF, Fish JA, Lee TK, Sun Y, Tiedje JM, et al. (2013). Ecological Patterns of nifH Genes in Four Terrestrial Climatic Zones Explored with Targeted Metagenomics Using FrameBot, a New Informatics Tool. mBio 4:e00592–13.

Xia W, Zhang C, Zeng X, Feng Y, Weng J, Lin X, et al. (2011). Autotrophic growth of nitrifying community in an agricultural soil. ISME J 5:1226–1236.

Zhalnina K, de Quadros PD, Gano KA, Davis-Richardson A, Fagen JR, Brown CT, et al. (2013). Ca. Nitrososphaera and Bradyrhizobium are inversely correlated and related to agricultural practices in long-term field experiments. Front Microbiol 4. doi:10.3389/fmicb.2013.00104.

Zumft WG. (1997). Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev 61:533–616.

SUPPLEMENTARY INFORMATION

Table S1 Number of sequences processed during bioinformatic analyses and number of normalized sequences used for calculation of diversity indexes and correlations.

Community (Gene)	Total number of sequence before bioinformatic analysis	Number of sequence per sample normalized for analyses
Bacteria (*16S rRNA*)	3050615	4312
Archaea (*16S rRNA*)	1861424	1185
AOB (*amoA*)	5100153	9982
AOA (*amoA*)	1371316	2914

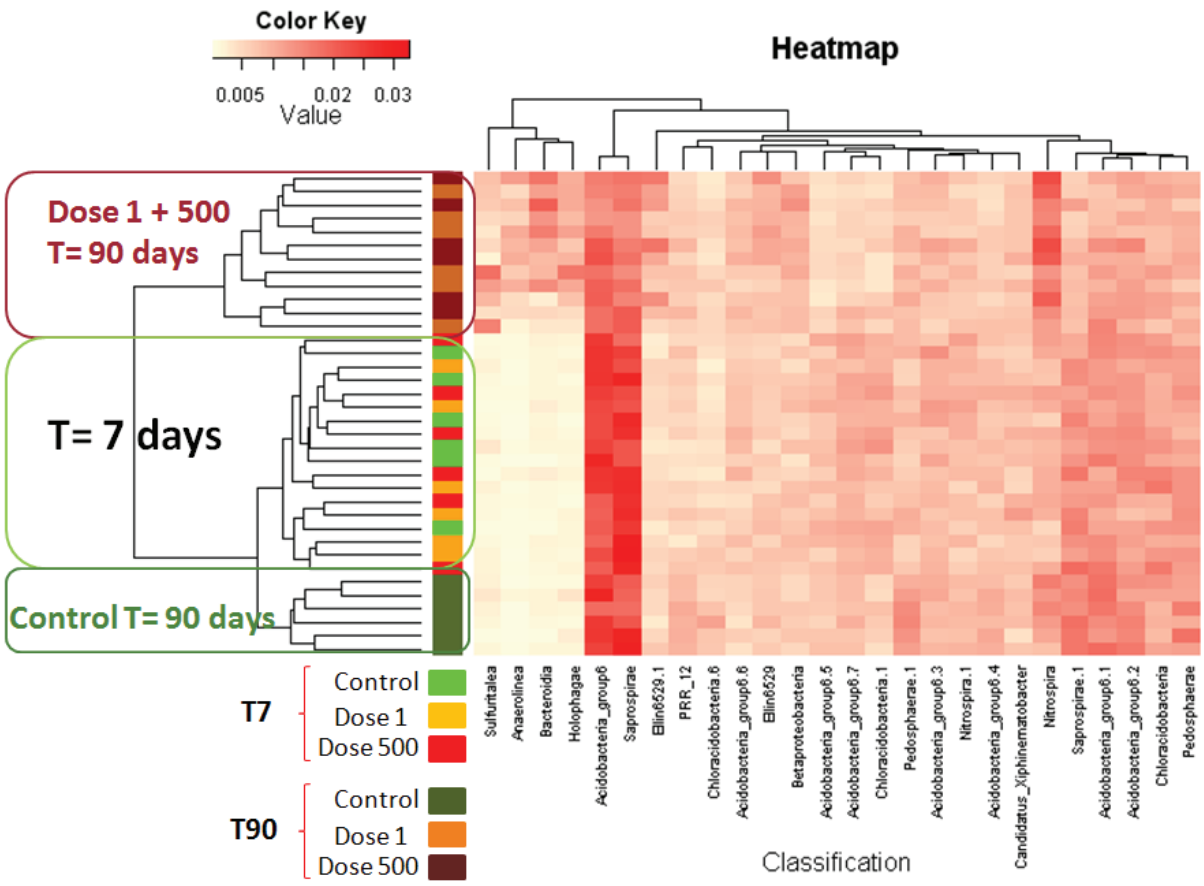


Figure S1 Relative abundance of bacterial OTUs in function of the different TiO_2-NPs concentrations and duration of incubation. The heatmap represents the proportions of OTUs at the genus level.

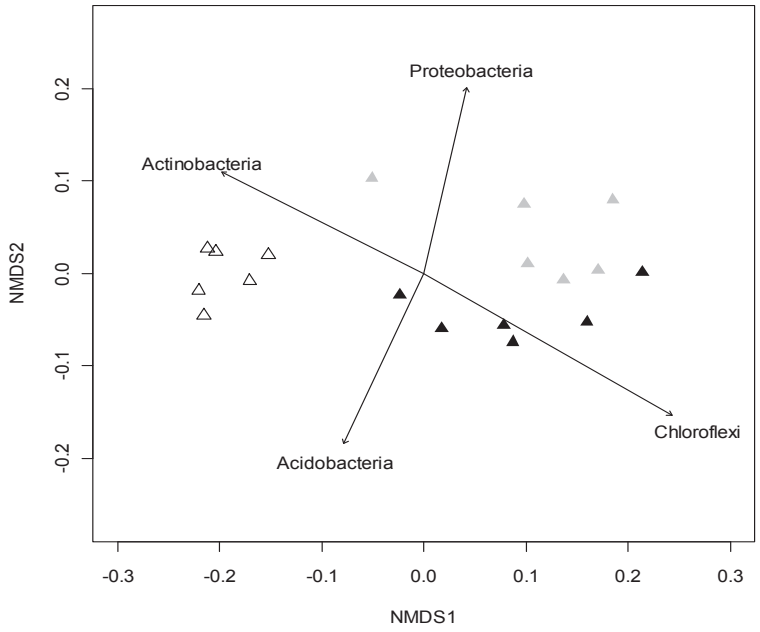


Figure S2 Nonmetric multidimensional scaling (NMDS) of the bacterial community structure after 90 days of exposure to TiO_2-NPs (control: white triangle; Dose 1: grey triangle; Dose 500: black triangle). Significant correlation between NMDS axis and bacterial phyla relative abundance, based on a post hoc permutation test (n = 999), are represented as arrows. The final stress for NMDS analysis was 0.06.

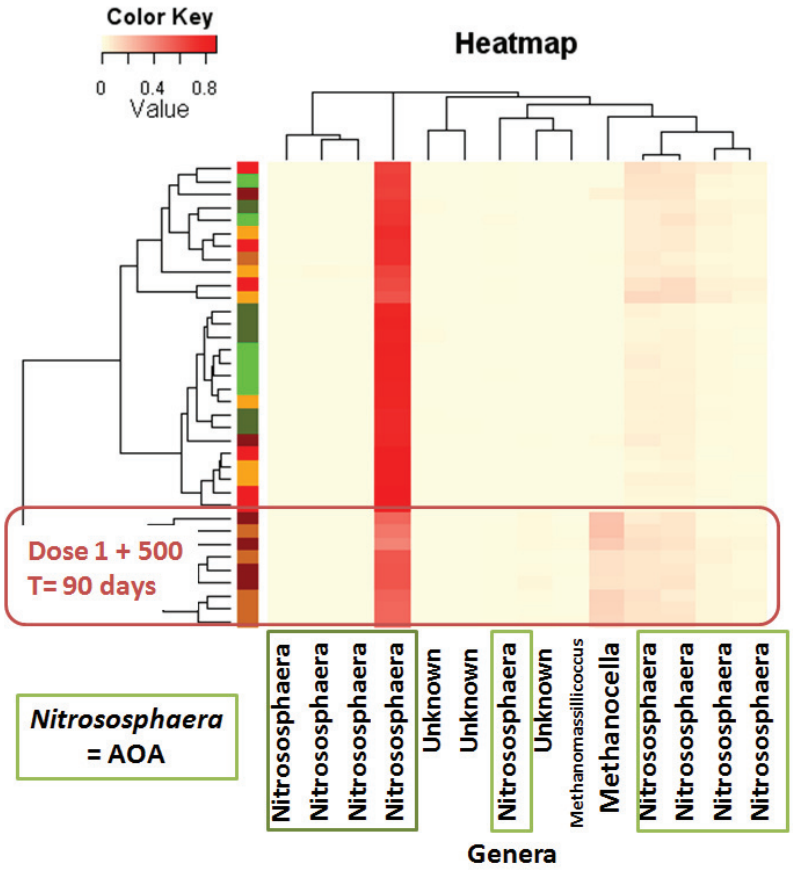


Figure S3 Relative abundance of archaeal genera in function of the different TiO_2-NPs concentrations and duration of incubation. The heatmap represents the proportions of OTUs at the genus level. See color legend on Figure S1.

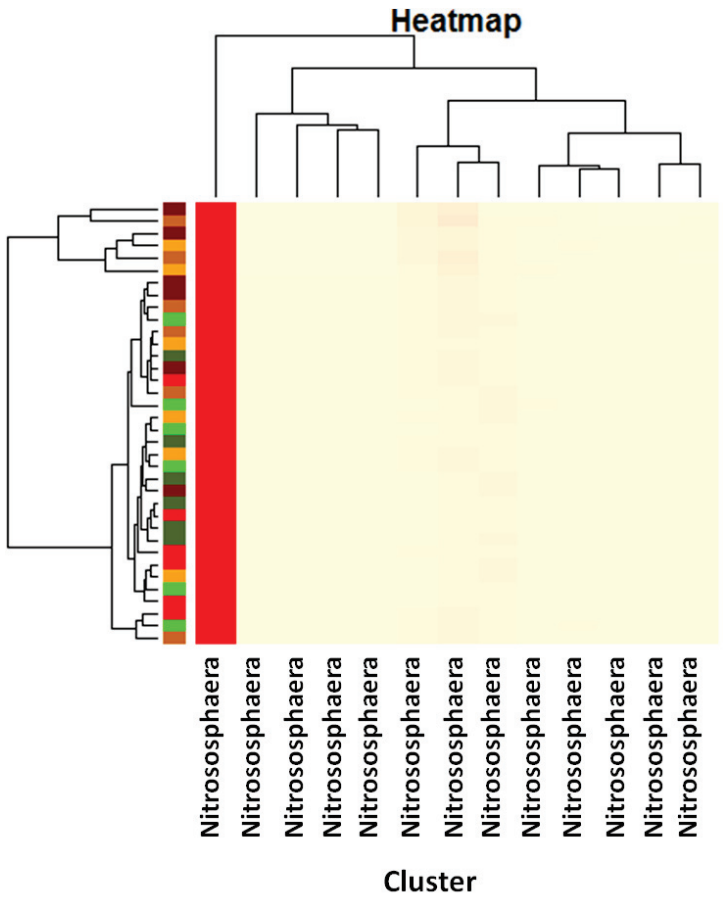


Figure S4 Relative abundance of AOA OTUs in function of the different TiO_2-NPs concentrations and duration of incubation. The heatmap represents the proportions of the different AOA clusters. See color legend on Figure S1.

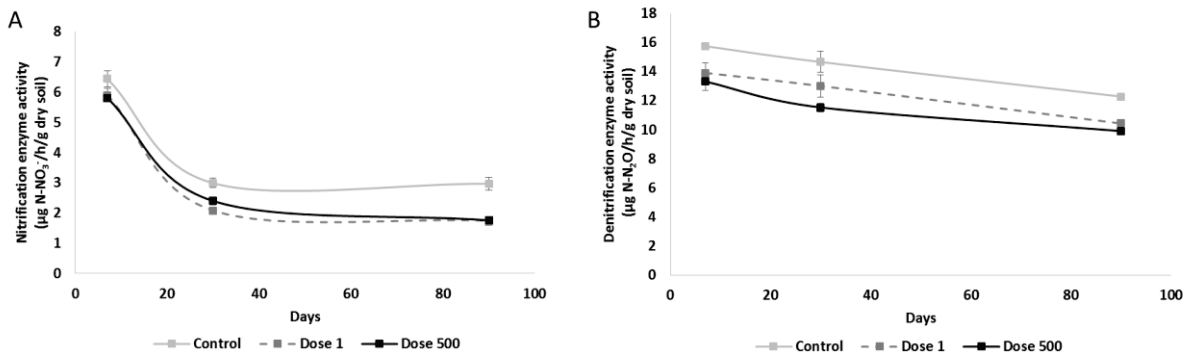


Figure S5 Dynamics over time of: A) NEA and B) DEA in the control (grey line) and in the Dose 1 mg kg^{-1} (dotted line) and Dose 500 mg kg^{-1} (black line) TiO_2 –NPs treatments.

3. Etude de l'effet dose-réponse du TiO_2 sur l'activité nitrifiante et les groupes fonctionnels nitrifiants lors d'une contamination aigüe

a. Article 5 : Présentation générale de l'étude et synthèse des principaux résultats

Dans l'article précédent, nous avons mis en évidence que la nitrification est un processus microbien sensible aux TiO_2-NPs dans le sol limono-argileux étudié. Nous avons observé que tout comme pour la minéralisation du carbone, des effets négatifs similaires étaient observés pour des concentrations de 1 ou 500 mg kg^{-1} sur les activités nitrifiantes et dénitrifiantes, ainsi que sur l'abondance des AOA. Cette absence de relation dose-réponse interpelle. C'est pourquoi nous avons approfondi cet aspect en évaluant l'influence d'une gamme de concentrations allant de 0,05 à 500 mg kg^{-1} sur la nitrification en utilisant le sol limono-argileux comme sol modèle. Cette expérience a permis de déterminer s'il existait une relation dose-réponse dans le cas d'une contamination aux TiO_2-NPs dans un sol, comme cela est observé pour des polluants solubles et utilisé pour déterminer par exemple des concentrations efficaces médianes (EC50) dans le cadre de réglementations. De plus, nous avons étudié l'effet des TiO_2-NPs à des concentrations très faibles, rarement évaluées dans la littérature (Article 1), qui peuvent représenter des doses réalistes dans les sols.

Les effets du TiO_2 aux différentes concentrations ont été évalués sur l'activité nitrifiante, ainsi que sur l'abondance des 4 groupes fonctionnels microbiens impliqués (AOA, AOB, NOB *Nitrobacter* et *Nitrospira*). Dans l'article précédent, seuls les effets sur les AOA et AOB impliqués dans la $1^{ère}$ étape de la nitrification avaient été considérés et 30 % de la variation de la nitrification n'avait pas été expliquée dans la path analysis. La seconde étape réalisée par les NOB *Nitrobacter* et *Nitrospira* n'est pas considérée comme limitante pour la nitrification (Kowalchuk and Schauss, 2001). Toutefois une accumulation du nitrite a été observée à plusieurs reprises dans des sols perturbés, suggérant que la seconde étape réalisée par les NOB peut parfois également être limitante (Roux-Michollet *et al.,* 2001 ; Ollivier *et al.,* 2013). C'est pourquoi dans cette étude, nous nous sommes également intéressés à la réponse des NOB aux TiO_2-NPs pour comparer les effets observés sur l'abondance des 4 groupes de nitrifiants et comprendre les conséquences sur l'activité nitrifiante globale. Grâce à une path analysis, nous avons déterminé l'influence de cette

perturbation sur le couplage entre les microorganismes oxydant l'ammonium (AOA ou AOB) et ceux oxydant le nitrite (NOB *Nitrobacter* et *Nitrospira*), afin d'expliquer les modifications d'activité nitrifiante en fonction des concentrations testées.

Afin de mieux comprendre la toxicité observée pour chaque concentration, l'agrégation et le potentiel oxydant (production de ROS) des TiO_2-NPs ont été mesurés. Ces paramètres sont connus pour être impliqués dans la biodisponibilité et la toxicité de ces composés (cf. chapitre 1).

Après 90 jours d'exposition aux différentes doses de TiO_2-NPs, nos résultats montrent des effets plus importants sur le groupe des AOA, alors que *Nitrospira* semble insensible à cette perturbation. L'abondance des AOB et des *Nitrobacter* a été modifiée de façon similaire et l'abondance de ces 2 groupes était fortement corrélée positivement. La nitrification potentielle a été réduite significativement à la concentration la plus faible (0.05 mg kg^{-1}) et aux concentrations les plus fortes (100 et 500 mg kg^{-1}).

Quelle que soit la variable mesurée, aucune relation dose-réponse linéaire n'a été observée et des pourcentages de diminution similaires ont été constatés pour des concentrations différant de plusieurs ordres de grandeur. Cette observation a pu être expliquée en partie par le fait que les caractéristiques physico-chimiques des TiO_2-NPs sont modifiées en fonction de la concentration utilisée. En particulier, nous avons pu mettre en évidence que l'agrégation du TiO_2 augmentait avec la concentration, alors que le potentiel oxydant diminuait. Ces résultats expliqueraient les diminutions significatives de l'activité nitrifiante ainsi que celles de l'abondance des AOA et *Nitrobacter* pour les concentrations testées les plus faibles (0.05 et 0.1 mg kg^{-1}). A ces faibles concentrations, les NPs sont moins agrégées et ont un potentiel oxydant plus grand ce qui très certainement leur confère une toxicité plus importante.

Via une approche par path analysis, nous avons montré que les altérations de la nitrification pouvaient être expliquées par des modifications de couplage entre les groupes fonctionnels impliqués, associées à une sensibilité différente de ces 4 groupes à cette perturbation. Ces résultats mettent en évidence une redondance fonctionnelle surprenante au sein des microorganismes oxydant l'ammonium et le nitrite, qui a permis de maintenir l'activité nitrifiante à des niveaux similaires à celui du contrôle pour la majorité des doses testées.

En conclusion, ces travaux mettent en évidence que les TiO_2-NPs peuvent avoir des effets délétères sur des processus microbiens clés à des concentrations très faibles dans les sols. Ils soulignent également la faible pertinence de l'utilisation des relations dose-réponses classiquement utilisées en écotoxicologie pour évaluer la toxicité des NPs dans les sols.

b. « Soil nitrification is altered by titanium dioxide nanoparticles through modifications of coupling between ammonia- and nitrite-oxidizers »

L'article 5 intitulé «Soil nitrification is altered by titanium dioxide nanoparticles through modifications of coupling between ammonia- and nitrite-oxidizers» a été soumis dans le journal *FEMS Microbiology Ecology* le 23 juin 2015.

Soil nitrification is altered by titanium dioxide nanoparticles through modifications of coupling between ammonia- and nitrite-oxidizers

Marie SIMONIN[1,2,3,4], Jean M. F. MARTINS[4], Aude CALAS[4], Gaëlle UZU[4], and Agnès RICHAUME[1,2,3*]

[1] Université de Lyon, Lyon, France

[2] Université Claude Bernard Lyon 1, Villeurbanne, France

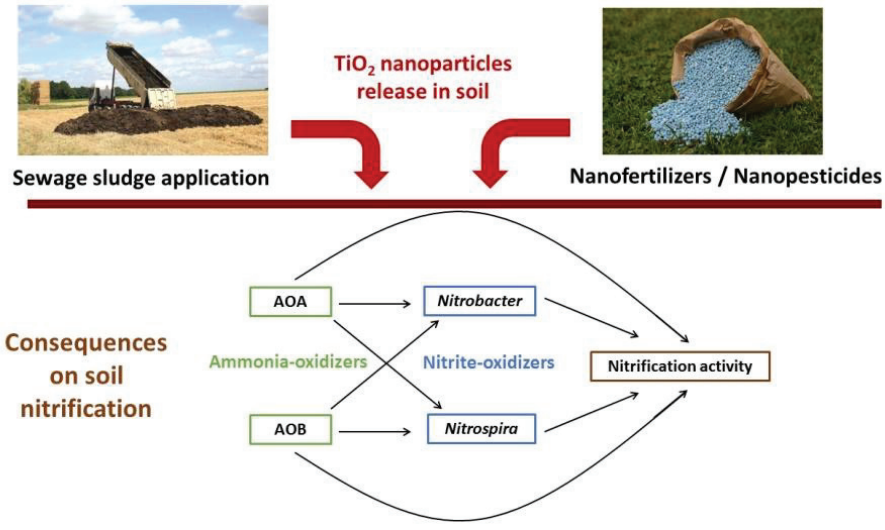
[3] CNRS, UMR 5557, Microbial Ecology, Université Lyon 1, Villeurbanne, France

[4] UJF-Grenoble/CNRS-INSU/G-INP/IRD, LTHE UMR 5564, Grenoble F-38041, France

[*]Correspondence to: agnes.richaume@univ-lyon1.fr

RUNNING TITLE **Soil nitrification sensitivity to TiO_2 nanoparticles**

GRAPHICAL ABSTRACT



ABSTRACT

Nitrification is a crucial microbiological process for soil fertility depending on the combined activity of ammonia- and nitrite-oxidizers. This key step of the N cycle is known to be very sensitive to environmental stressors. However, the impact of emerging pollutants such as titanium-dioxide nanoparticles (TiO_2-NPs) which are increasingly released in agricultural soils, remains to be investigated.

We assessed the impact of eight TiO_2-NPs concentrations (0.05-500 mg kg^{-1} dry-soil) in soil microcosms on potential nitrification activity and abundance of ammonia-oxidizing archaea (AOA), bacteria (AOB) and nitrite-oxidizers (*Nitrobacter* and *Nitrospira*) using quantitative PCR. In addition, aggregation and oxidative potential of TiO_2-NPs were measured to identify the main drivers of TiO_2-NPs toxicity in soil.

After 90 days of exposure, AOA appeared to be the most affected by TiO_2-NPs, while *Nitrospira* seemed insensitive to this contamination. AOB and *Nitrobacter* abundance exhibited similar responses. Interestingly, the potential nitrification activity was reduced by the lowest (0.05 mg kg^{-1}) and the highest (100 and 500 mg kg^{-1}) TiO_2-NPs concentrations. A path analysis suggested that the alteration of nitrification could be explained by changes in the coupling between ammonia- and nitrite-oxidizers triggered by a different sensitivity of these microbial groups to this contaminant.

Key-words: TiO_2 Nanomaterials, Microbial Ecotoxicology, Nitrification, Structural Equation Modeling

INTRODUCTION

Soils are constantly submitted to new environmental stressors, such as metal-oxide nanoparticles (NPs), which are worrying emerging pollutants. Metal-oxide NPs are present in many commercial products (food, cosmetics, paints, electronics…) and can be released in the environment all along their life cycle from production to disposal (Mitrano et al., 2015). Soils are particularly exposed to NPs that can be found in sewage sludge used as agricultural amendments or in nanofertilizers and nanopesticides (Brar et al., 2010; Servin et al., 2015). Titanium-dioxide (TiO_2) NPs are the most produced NPs and it is predicted that they would represent 50% of all NPs retrieved in soil (Keller et al., 2013; Sun et al., 2014). The few available studies have shown that TiO_2-NPs could have detrimental effects on soil microbial communities, as deduced from the decrease of soil respiration or bacterial diversity and modifications of soil bacterial community structure (Ge et al., 2011, 2012; Simonin et al., 2015a). In a recent review, we identified several research needs on NPs ecotoxicity (Simonin and Richaume, 2015). Among these, the assessment of their impact using a range of relevant environmental TiO_2-NPs concentrations is of interest since traditional toxicological assays to find out dose–response relationships are not workable in the case of nanoparticles (Editorial Nature Nanotechnol 2011). Given bacteria are recognized as important targets to consider when assessing NPs impact in soils, the consequence of NPs toxicity on microbial functional groups involved in key soil services such as fertility, is relevant in risk assessment studies. In this context, the microbial functional groups involved in the N cycle, especially nitrification, appear to be an ideal model (Wessén and Hallin, 2011; Pereira e Silva et al., 2012). Indeed, one can assume that the effect of NPs on microbial functional groups exhibiting low diversity and low functional redundancy such as nitrifiers could be detrimental to soil functioning.

Nitrification is crucial for plant nutrition and biomass production by controlling soil inorganic-N availability. This is also one of the most sensitive microbial process to disturbance, such as pollutant contamination (Dalzell et al., 2002; Broos et al., 2005). Nitrification is a two-step process performed by phylogenetically constrained groups of microorganisms. Ammonia-oxidation (oxidation of NH_4^+ in NO_2^-) is carried out by ammonia-oxidizing archaea (AOA) (Leininger et al., 2006) and ammonia-oxidizing bacteria (AOB)

(Kowalchuk and Stephen, 2001). Most of the literature about nitrification is focused on this step, as it is considered as the limiting-step of the whole nitrification (Kowalchuk and Stephen, 2001). Although AOA were discovered recently (Könneke et al., 2005), their functional role in nitrification is now well established (Prosser and Nicol, 2012; Zhalnina et al., 2012). Nevertheless, little knowledge is available about their sensitivity to environmental stressors (global change, pollutants…) (Mertens et al., 2009; Ruyters et al. 2010; Ollivier et al., 2012).

The second step of nitrification is nitrite-oxidation (oxidation of NO_2^- in NO_3^-) which is performed by nitrite-oxidizing bacteria (NOB), especially the *Nitrobacter* and *Nitrospira* genera in soil (Freitag et al., 2005). Compared to ammonia-oxidation, nitrite-oxidation is less studied, although efficient nitrification activity requires the presence of both ammonia-oxidizers and nitrite-oxidizers (Ollivier et al., 2013). In disturbed soils, nitrite can accumulate indicating that nitrite-oxidation could be the limiting-step of nitrification in some cases (Gelfand and Yakir, 2008; Roux-Michollet et al., 2008). However, nowadays the responses of AOA and NOB to stressors and the alteration of the coupling between ammonia-oxidizers and nitrite-oxidizers in disturbed soil systems is insufficiently documented (Ollivier et al., 2013).

In vitro NPs toxicity is attributed to an oxidative stress associated to the production of reactive oxygen species (ROS) from NPs in contact with microbial membranes, leading to membranes disruption, proteins oxidation, or energy transduction interruption (Klaine et al., 2008; Neal, 2008; Xia et al., 2008). However, these drivers of NPs toxicity have not been demonstrated in soil yet. This can be explain by (i) current technical limitations to detect NPs in complex media (Tourinho et al., 2012; Cornelis et al., 2014), (ii) the multiple and complex physicochemical transformations that NPs can undergo in soil, such as homo- and heteroaggregation, which are greatly influenced by environmental abiotic factors (e.g. pH, ionic strength, organic matter) (French et al., 2009; Keller et al., 2010; Lowry et al., 2012), (iii) the influence of NPs concentration on their physicochemical properties, especially on their aggregation and reactivity (Menard et al., 2011; Vitorge et al., 2013; Pokhrel et al., 2014). Thus, to date it is impossible to predict the impact of different NPs concentrations on soil organisms and little is known on the toxicological mechanisms involved.

In this study, we assessed the impact of a range of 8 TiO_2-NPs concentrations varying from 0.05 to 500 mg kg^{-1} dry soil on the potential nitrification activity and the abundance of ammonia-oxidizers (AOA and AOB) and nitrite-oxidizers (NOB *Nitrobacter* and *Nitrospira*) using quantitative PCR. Concentrations ranging between 0.05 and 0.5 mg kg^{-1} were considered as low concentrations; those ranging between 1 and 50 mg kg^{-1} as intermediate concentrations and 100 and 500 mg kg^{-1} as high concentrations. The experiment was performed with a silty-clay soil that was artificially contaminated in the laboratory and exposed to TiO_2-NPs for 90 days in microcosms. We assume that the responses to TiO_2-NPs of AOA, AOB, *Nitrobacter* and *Nitrospira* could differ because of their different physiology and ecology. A path analysis was used to determine if the effects on potential nitrification could be explained by the modification of nitrifier abundance and if the functional drivers of nitrification and coupling between ammonia- and nitrite-oxidizers were modified according to the TiO_2-NPs concentrations. In addition, we measured the aggregation and oxidative potential (total ROS production) of TiO_2-NPs in suspension in water versus soil solution at different concentrations. These data have been collected in order to identify and understand better the main drivers of TiO_2-NPs toxicity in soil and the effects on nitrification activity and nitrifier abundance.

MATERIALS AND METHODS

TiO_2 Nanoparticles

Titanium dioxide nanoparticles (TiO_2-NPs) were provided by Sigma Aldrich (St Louis, USA) (mixture of anatase (80%) and rutile (20%) crystal structure) with at least 99.5% purity. According to the manufacturer information, TiO_2-NPs presented a specific surface area of 35-65 $m^2.g^{-1}$ and a mean particle size of 21 nm in powder as measured by Transmission Electron Microscopy.

Soil microcosms and experimental design

Soil was collected from the upper 20 cm layer of a silty-clay soil (Cambisol, WRB, 2006) sampled at Commarin (Côte d'Or, France) under a permanent pasture. After collection, roots

and plant litter were manually removed. The soil was sieved (2 mm) and homogenized before storage at 4°C. The soil main characteristics were: sand 10%; loam 51%; clay 39%; pH 7.7; OM 7.9%; CEC 20.1 cmol kg^{-1}; Water holding capacity (WHC) 51%.

This soil was exposed to eight increasing TiO_2-NPs concentrations in microcosms for 90 days in the dark at 28°C. One kg of soil (equivalent dry weight – eq dw) was spiked with a defined volume of TiO_2-NPs suspensions prepared in ultrapure water (0.625 to 6250 mg TiO_2 L^{-1}) in order to obtain 0.05 to 500 mg TiO_2 kg^{-1} dry soil (Table 1) and to increase the soil water content to the WHC. These concentrations were chosen to represent different exposures from a very low realistic environmental concentration (0.05 mg kg^{-1} dry soil) to a high concentration representing an accidental spill (500 mg kg^{-1} dry soil) (Sun et al., 2014). The TiO_2-NPs suspensions were added homogeneously to soil using a multichannel pipette and then soils were thoroughly mixed for 10 minutes to ensure a uniform spiking. Soils receiving only the same volume of ultrapure water were used as controls. Microcosms were set up by placing 50 g of control or spiked soils (eq dw) into 150 mL glass plasma flasks sealed with rubber stoppers to avoid soil drying and to maintain the soil moisture constant during the experiment. Microcosms were weekly aerated for 5 minutes to ensure a renewal of the atmosphere in the flasks. This experimental design resulted in a total of 36 microcosms with 4 replicates per treatment. At the end of each incubation time, microcosms were subsampled as follows: 3 g of soil (eq dw) were immediately used for the measurements of potential nitrification, 3 g of soil were stored at -20°C before DNA extraction.

Table 1 Correspondences between TiO_2 concentration in spiking suspensions and the final TiO_2 concentration in soil microcosms

Concentration TiO_2 :	**in spiking suspension (mg L^{-1})**	0.625	1.25	6.25	12.5	125	625	1250	6250
	in soil (mg kg^{-1} dry soil)	0.05	0.1	0.5	1	10	50	100	500

Soil solution preparation

Soil solutions were prepared following the protocol described by Simonin et al. (2015a) to characterize TiO_2-NPs in physicochemical conditions as close as possible to those

encountered in soil in terms of pH, ionic strength and dissolved components. Soil colloids larger than the initial size of TiO_2-NPs (i.e. 20 nm) were removed from the suspension in order to get a reliable measurement of NPs aggregation and oxidative potential. Briefly, soil solutions were prepared by shaking 10 g of uncontaminated soil dispersed in 50 mL of ultrapure water during 30 min (180 rpm, 20°C) in a refrigerated incubator shaker (New Brunswick – Eppendorf, Hamburg, Germany). The solutions were then centrifuged for 20 min (8000 g, 20°C, Centrifuge 5804R, Eppendorf, Hamburg, Germany) and the supernatants were collected and stored at 4°C.

Aggregation and oxidative potential of TiO_2-NPs

TiO_2-NPs suspensions were prepared in ultrapure water or in soil solutions at different concentrations ranging from 0.625 to 125 mg L^{-1}. Higher concentrations of TiO_2-NPs were tested but did not allow to obtain reliable measurements of aggregation or oxidative activity, because of sample fluorescence and coloration. All TiO_2-NPs suspensions were dispersed through ultrasonication for 5 min before measurements to ensure suspension homogeneity.

The apparent hydrodynamic diameter of TiO_2-NPs in ultrapure water or soil solutions was determined by dynamic light scattering (DLS) using a NanoZS Zetasizer (Malvern Instruments –UK) as described in Vitorge et al. (2014). For each suspension, the mean of 3 measurements was recorded.

The oxidative potential of TiO_2-NPs in ultrapure water and soil solution was assessed using the 2',7'-dichlorofluorescein (DCFH) assay, following Foucaud et al (2007). DCFH is a non-fluorescent reagent that becomes fluorescent dichlorofluorescin (DCF) when oxidized in the presence of ROS.

DCFH was prepared from 2',7'-dichlorofluorescein diacetate (DCFH-DA) according to the method provided by Cathcart et al. (1983). We mixed 0.5 mL of 1 mM DCFH-DA in methanol with 2 mL of 0.01M sodium hydroxide. The hydrolysate was allowed to stand at room temperature for 30 min and then neutralized with 10 mL phosphate buffer (KH_2PO_4, 0.1M). The solution was kept on ice avoiding any light exposure until use.

The measurement of DCFH oxidation by TiO_2-NPs was performed in 48-wells plates in which were added: 200 µL of DCFH and 600 µL of the different TiO_2-NPs suspensions prepared in

ultrapure water or in soil solution. Ultrapure water or soil solution was used as negative control and a 0.5M H_2O_2 solution was used as positive control. All assays were performed in triplicate. The fluorescence generated by the DCFH oxidation by ROS was measured in a microplate reader (Tecan Infinite M200) at 485 nm excitation and 530 nm emission, every 10 minutes during one hour.

DNA extraction and quantification of the abundance of nitrifiers

DNA was extracted from 0.5 g of frozen soil using the Power Soil™ DNA Isolation Kit (MO BIO laboratories, Carlsbad, CA, USA), following the manufacturer's instructions and then DNA concentrations were determined using the Qubit dsDNA BR Assay (Invitrogen).

The abundance of the ammonia-oxidizers (AOA and AOB) and nitrite-oxidizers (*Nitrobacter* and *Nitrospira*) were measured by quantitative PCR using a Lightcycler 480 (Roche Diagnostics, Meylan, France). For AOA and AOB quantification, the final reaction volume was 20 µL and contained (final concentrations) 0.5 µM of each primer for the bacterial *amoA* or 0.75µM of CrenamoA616r and 1µM of CrenamoA23f for the archaeal *amoA*, 2% bovine serum albumin (BSA), 1X of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 10 ng of soil DNA extract or 10^7-10^2 gene copies number μL^{-1} of an in-house plasmid containing cloned bacterial (*Nitrosomonas europaea*, GenBank accession number:L08050) and archaeal (54d9 fosmide fragment, Treusch et al., 2005) *amoA* genes.

For *Nitrobacter*-like NOB quantification, the final reaction volume was 20 µL and contained 0.5 µM of each primer, 1X of QuantiTect SybrGreen PCR Master Mix and 40 ng of soil DNA extract or 10^7-10^2 copies using a linearized plasmid containing cloned *nxrA* gene of *Nitrobacter hamburgensis* X14 (DSMZ 10229).

For *Nitrospira* quantification the final reaction volume was 25 µL and contained (final concentrations) 0.4 µM of each primer, 1X of QuantiTect SybrGreen PCR Master Mix and 10 ng of soil DNA extract or 10^7-10^2 *Nitrospira* copies of linearized plasmid DNA (GenBank accession number: FJ529918). All primers and thermal cycling conditions used are described in Table 2.

Melting curve analysis confirmed the specificity of amplification of the four genes. High amplification efficiencies of 96–98% were obtained.

Table 2 PCR primers and thermal cycling conditions used for quantification of ammonia- and nitrite-oxidizer abundance

Primers	Target gene	Reference	Thermal conditions
CrenamoA23f	AOA *amoA*	Tourna et al. 2008	15 min at 95°C, 45 cycles (45 s at 94°C, 45 s at 55°C and 45 s at 72°C)
CrenamoA616r	AOA *amoA*	Tourna et al. 2008	
amoA-1F	AOB *amoA*	Rotthauwe et al. 1997	15 min at 95°C, 45 cycles (30 s at 95°C, 45 s at 54°C, 45 s at 72°C and 15 s at 80°C)
amoA-2R	AOB *amoA*	Rotthauwe et al. 1997	
F1norA	NOB *Nitrobacter nxrA*	Poly et al. 2008	15 min at 95°C, 45 cycles (30 s at 95°C, 45 s at 55°C and 45 s at 72°C)
R2norA	NOB *Nitrobacter nxrA*	Wertz et al. 2008	
Ns675f	NOB *Nitrospira 16S rRNA*	Graham et al. 2007	15 min at 95°C, 45 cycles (30 s at 95°C, 30 s at 66°C and 1 min at 72°C)
Ns746r	NOB *Nitrospira 16S rRNA*	Graham et al. 2007	

Nitrification activity

Potential nitrification activity was determined following the protocol described in Dassonville et al. (2011). Sub-samples of fresh soil (3 g equivalent dry soil) were incubated with 6 ml of a solution of $(NH_4)_2SO_4$ (50 µg N-NH_4^+ g^{-1} dry soil). Distilled water was added in each sample to reach 24 ml of total liquid volume in flasks. The flasks were sealed with Parafilm® and incubated at 28°C with constant agitation (180 rpm). During incubation, 1.5 ml of soil slurry was sampled at 2h, 4h, 6h, 8h and 10h, filtered (0.2 µm pore size) and transferred into vials. Samples were stored at -20°C until analysis of NO_3^- concentrations by ionic chromatography (DX120, Dionex, Salt Lake City, USA) equipped with a 4 mm×250 mm column (IonPac AS9 HC). Potential nitrification was expressed as µg N-NO_3^- h^{-1} g^{-1} dry soil.

Statistical analysis

All results are presented as means (± standard error). A one-way analysis of variance (ANOVA) and *post-hoc* Tukey HSD were performed to test the effect of TiO_2-NPs concentrations on measured variables. When necessary, data were log-transformed prior to analysis to ensure conformity with the assumptions of normality and homogeneity of variances. Linear regressions between all variables measured were investigated. Effects with

P<0.05 are referred to as significant. These statistical analyses were carried out using R statistical software 2.13.2 (R Core Team, 2015).

Path analysis was performed using Amos18® (Amos Development Corporation, Crawfordville, FL, USA) to explore the causal links between nitrifier abundance (AOA, AOB, *Nitrobacter* and *Nitrospira*) and nitrification rates (Shipley, 2002). This path analysis enabled to determine which groups of ammonia-oxidizers and nitrite-oxidizers are the main functional drivers of nitrification in our soil and if the drivers of nitrification are modified in function of the TiO_2-NPs concentration applied.

Four path analysis were performed using different sets of data: i) all concentrations of TiO_2-NPs, ii) only low concentrations of TiO_2-NPs (0.05; 0.1 and 0.5 mg kg^{-1}), iii) only intermediate concentrations (1; 10; 50 mg kg^{-1}) and iv) only high concentrations of TiO_2-NPs (100 and 500 mg kg^{-1}). A χ^2 test was used to evaluate model fit by determining whether the covariance structures implied by the model adequately fit the actual covariance structures of the data. The coefficients of each path as the calculated standardized coefficients were determined using the analysis of correlation matrices. These path coefficients indicate how many standard deviations the effect variable would change, if the causal variable was changed by one standard deviation. Paths in this model were considered significant with a *P*< 0.05.

RESULTS

Aggregation and oxidative potential of TiO_2-NPs

Aggregation and oxidative potential (OP) of TiO_2-NPs were characterized in the aqueous suspensions used to spike the soil (i.e. in ultrapure water) and in soil solutions prepared at TiO_2-NPs concentrations ranging from 0.625 to 125 mg L^{-1}.

In both ultrapure water and soil solutions, TiO_2-NPs aggregation measured by Dynamic Light Scattering (DLS) increased with NPs concentration (Fig. 1A). TiO_2-NPs were more aggregated (i.e. higher apparent hydrodynamic diameter) in ultrapure water than in soil solution (126 to 173 nm vs 86 to 153 nm, Fig. 1A) in case of concentrations ranging from 0.625 to 50 mg L^{-1}. At the highest concentrations tested (100 and 125 mg L^{-1}), TiO_2-NPs presented a strong and

similar aggregation with an average apparent diameter of about 160 nm in both ultrapure water and soil solution.

The OP of TiO_2-NPs measured in soil solution and ultrapure water assessed by DCFH oxidation was maximum for the lowest concentration tested (0.625 mg L^{-1}) and decreased progressively with increasing TiO_2-NPs concentration (Fig. 1B). The oxidative activity of TiO_2-NPs was higher in soil solution than in ultrapure water for all concentrations tested except for the 12.5 mg L^{-1} (Fig. 1B). In soil solution, the OP of TiO_2-NPs ranged from 35 to 0.2 fluorescence units sec^{-1} µg TiO_2^{-1} between 0.625 and 125 mg L^{-1}, while in ultrapure water the OP ranged from 5 to 0.02 fluorescence units sec^{-1} $µg^{-1}$ TiO_2.

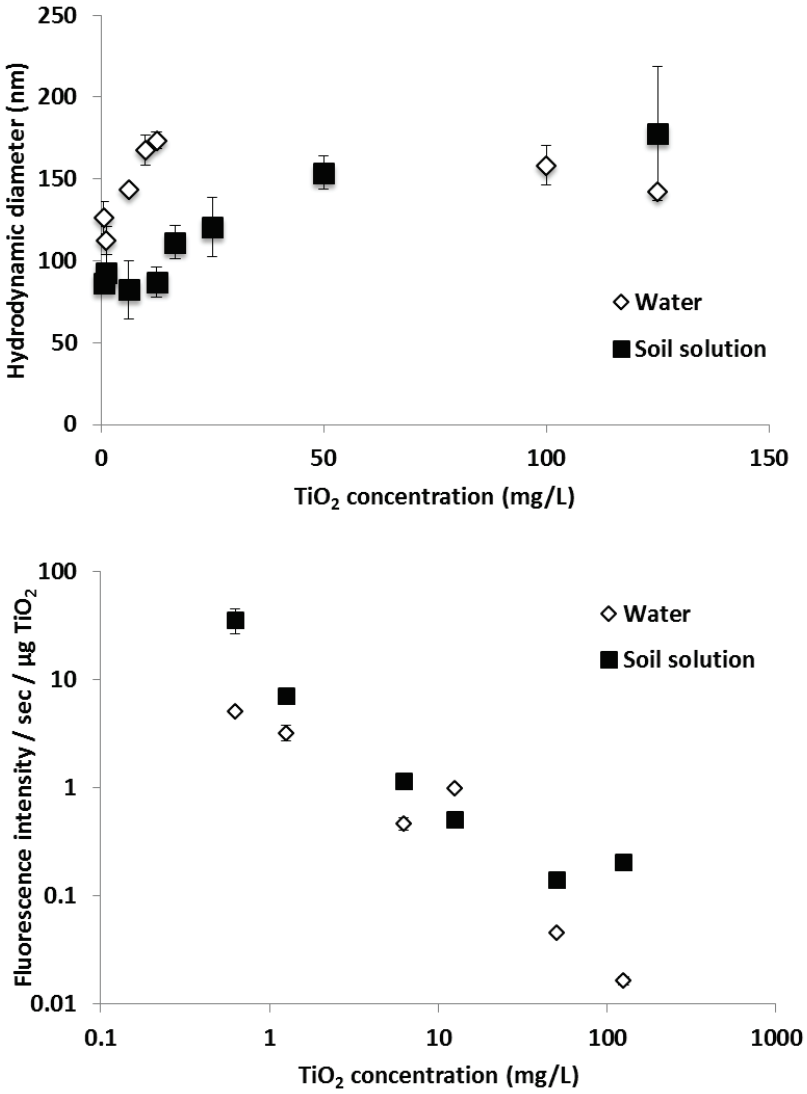


Figure 1 Influence of TiO_2-NPs concentration in ultrapure water or soil solution on A) the aggregation of TiO_2-NPs measured by Dynamic Light Scattering and B) the oxidative potential

(OP) assessed by DCFH oxidation and expressed as Fluorescence intensity (Arbitrary units) per second and per µg of TiO_2-NPs.

Ammonia- and nitrite oxidizer abundance

In this soil, among ammonia-oxidizers, the AOA were dominant over AOB (54 fold higher abundance on average) and among nitrite-oxidizers, the *Nitrospira* genus was dominant (441 fold higher abundance) over *Nitrobacter*.

After 90 days of exposure in microcosms, AOA abundance was similarly reduced (around -40%) by low (Fig. 2A, 0.1 mg kg^{-1}; 0.5 mg kg^{-1}), intermediate (10 mg kg^{-1}) or high TiO_2-NPs concentrations (100 mg kg^{-1}; 500 mg kg^{-1}). The concentrations 0.05, 1 and 50 mg kg^{-1} lowered AOA abundance (-30 to -35%) but these effects were found not significant because of a high variability of these qPCR data. In contrast, AOB abundance was significantly decreased only by intermediate concentrations of TiO_2-NPs (Fig. 2B, 1 mg kg^{-1}: -32%; 10 mg kg^{-1}: -29%). Low and intermediate concentrations of TiO_2-NPs reduced *Nitrobacter*-like NOB abundance (Fig. 2C, 0.1 mg kg^{-1}: -28%; 0.5 mg kg^{-1}: -32%; 1 mg kg^{-1}: -33%; 10 mg kg^{-1}: -36%). A marginally significant reduction was also observed for a high TiO_2-NPs concentration (100 mg kg^{-1}: -30%). On the contrary, no significant was found on *Nitrospira* abundance whatever the concentration (Fig. 2D).

Table 3 Correlations between nitrifiers abundance (AOA, AOB, *Nitrobacter*-like NOB and *Nitrospira*) on all data (n=36). The R^2 values are given and significant correlations (*, $P<0.05$; ***, $P<0.001$) are presented in bold.

	AOA	**AOB**	***Nitrobacter***
AOB	0.09		
Nitrobacter	**0.18 ***	**0.75 *****	
Nitrospira	0.11	**0.42 *****	**0.36 *****

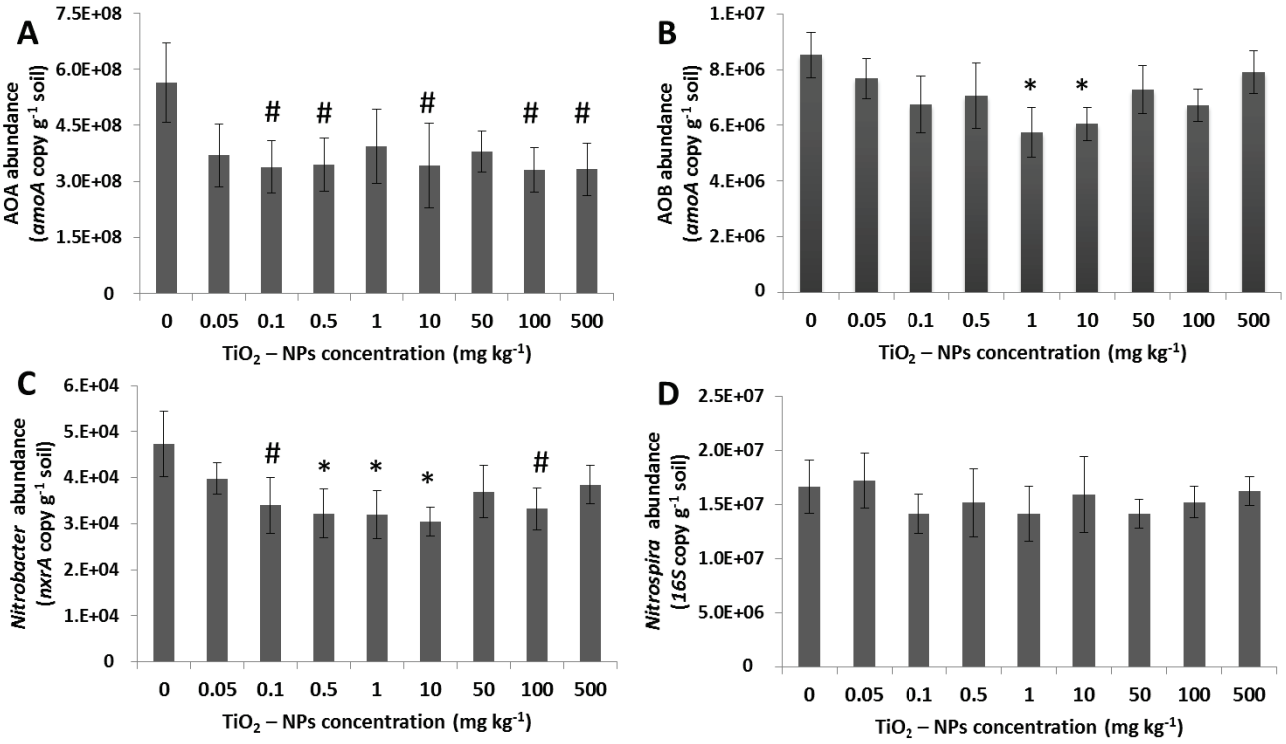


Figure 2 Effect of the different concentrations of TiO_2-NPs after 90 days of exposure on abundances of: A) AOA, B) AOB, C) *Nitrobacter*-like NOB, D) *Nitrospira*. Means and standard errors are presented (n=4). Significant effects and marginally significant effects are indicated (#, P<0.1; *, P<0.05).

The abundance of AOA and AOB was not correlated, whereas the abundance of *Nitrobacter*-like NOB and *Nitrospira* was positively correlated (Table 3). A strong correlation was found between *Nitrobacter*-like NOB and AOB abundance, while *Nitrobacter*-like NOB abundance was weakly correlated to AOA abundance (Table 3). Similarly, *Nitrospira* abundance was positively correlated to AOB abundance, but not AOA abundance (Table 3).

Nitrification activity

After 90 days of exposure in microcosms, soil potential nitrification was significantly decreased by 25% and 26% by the lowest concentration of TiO_2-NPs (Fig. 3) and the two

highest concentrations, respectively (100 mg and 500 mg kg^{-1}). The other concentrations ranging from 0.1 to 50 mg kg^{-1} had no effect on the nitrification activity.

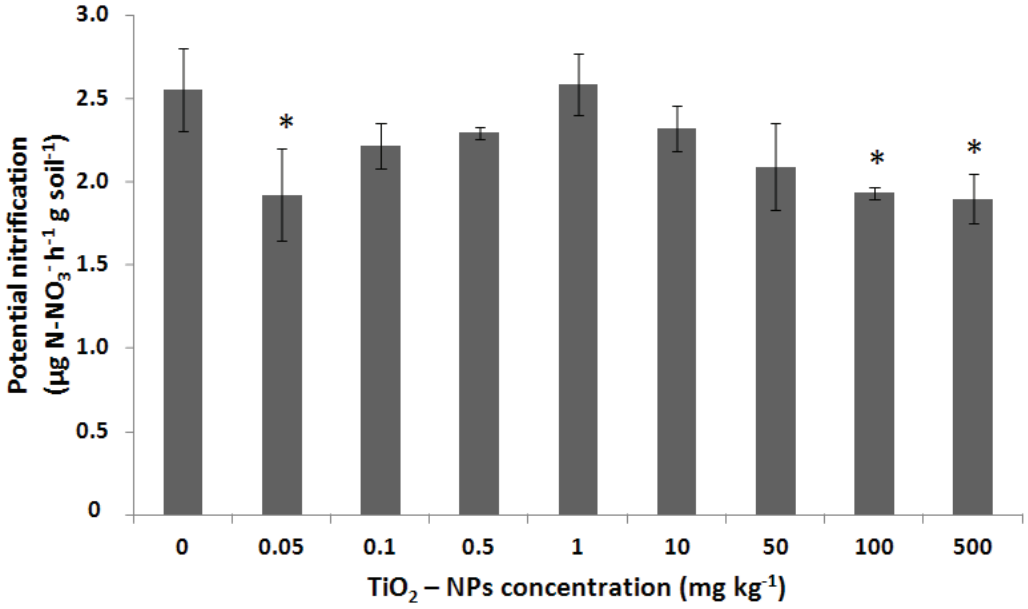


Figure 3 Effect of increasing concentrations of TiO_2-NPs on soil potential nitrification activity measured after 90 days of exposure. Means and standard errors are presented (n=4). Significant effects are indicated (*, P<0.05).

Potential nitrification was not correlated to any of the nitrifier abundance measured (data not shown) when considering the entire data set (n=36). However, a positive correlation was found between nitrification activity and AOA abundance in the samples exhibiting a significantly reduced nitrifying activity (concentrations 0.05, 100 and 500 mg kg^{-1}, Fig. 4).

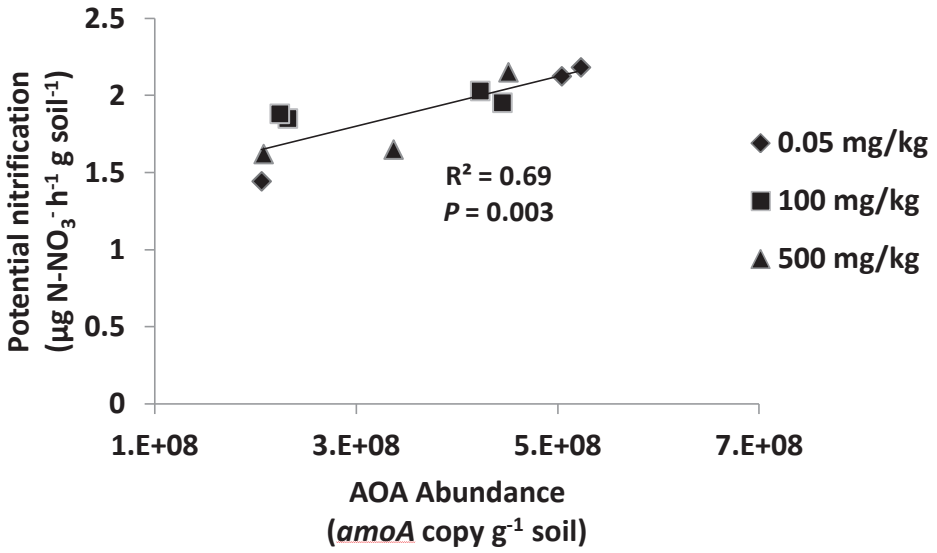


Figure 4 Correlation between potential nitrification and AOA abundance in the samples spiked with 0.05, 100 and 500 mg kg^{-1}.

Path analysis

In order to determine whether the effects of TiO_2-NPs on potential nitrification could be explained by the modification of nitrifier abundance and of the coupling between ammonia- and nitrite-oxidizers, we used a path analysis approach. These path analysis were performed based on a theoretical full model (Fig. 5A), which includes all possible relationships between ammonia-, nitrite-oxidizer abundance and potential nitrification activity. Final models with the best fit were obtained after removing the paths that did not contribute to explain the variations of nitrification or NOB abundance in the model.

When considering the complete data set of the TiO_2-NPs concentrations tested, the path analysis enabled to explain a low part of the variation of the nitrification activity (Fig. 5B, R^2=16%). The variables that explained the largest part of potential nitrification variation were AOB and *Nitrobacter* abundance (AOB: path coefficient=-0.54, *P*=0.06; *Nitrobacter*: path coefficient=0.18, *P*=0.53). In addition, *Nitrobacter* abundance was mainly controlled by AOB abundance (path coefficient=0.85, *P*<0.001) and more weakly by AOA abundance (path coefficient=0.17, *P*=0.03), explaining a total variation of 75% of the *Nitrobacter* data.

We also performed a path analysis based on 3 different subsamples of the data set according to the TiO_2-NPs concentration (i.e low, intermediate or high concentrations, Fig. 5C, 5D and 5E). In the low concentration data set (Fig. 5C), the path analysis indicated that AOA and *Nitrospira* abundance explained 94% of nitrification variation (AOA: path coefficient=0.21, *P*=0.008; Nitrobacter: path coefficient=-0.95, *P*<0.001). This model also showed that AOB abundance was an important predictor of *Nitrospira* abundance (R^2=0.87, path coefficient= 0.93, *P*<0.001).

In the intermediate concentration data set (Fig. 5D), the four nitrifier groups were directly (*Nitrobacter* and *Nitrospira*) or indirectly related (AOA and AOB) to potential nitrification and explained 41% of nitrification variation. AOA abundance influenced *Nitrospira* (R^2=0.45, path coefficient=0.67, *P*=0.045) and *Nitrobacter* abundance (path coefficient= 0.25, *P*=0.002). However, AOB abundance was the most important predictor of *Nitrobacter* abundance (path coefficient= 0.88, *P*<0.001).

In the high concentration data set (Fig. 5E), the most important explanatory variable of nitrification activity was AOA abundance (path coefficient=0.70, *P*=0.002) followed by *Nitrobacter* abundance (path coefficient=-0.28, *P*=0.22), explaining 67% of variation in the nitrification data. Furthermore, *Nitrobacter* abundance was mainly controlled by AOB abundance (path coefficient=0.87, *P*<0.001) and more weakly by AOA abundance (path coefficient=-0.25, *P*=0.13), explaining a total variation of 75% of the *Nitrobacter* data.

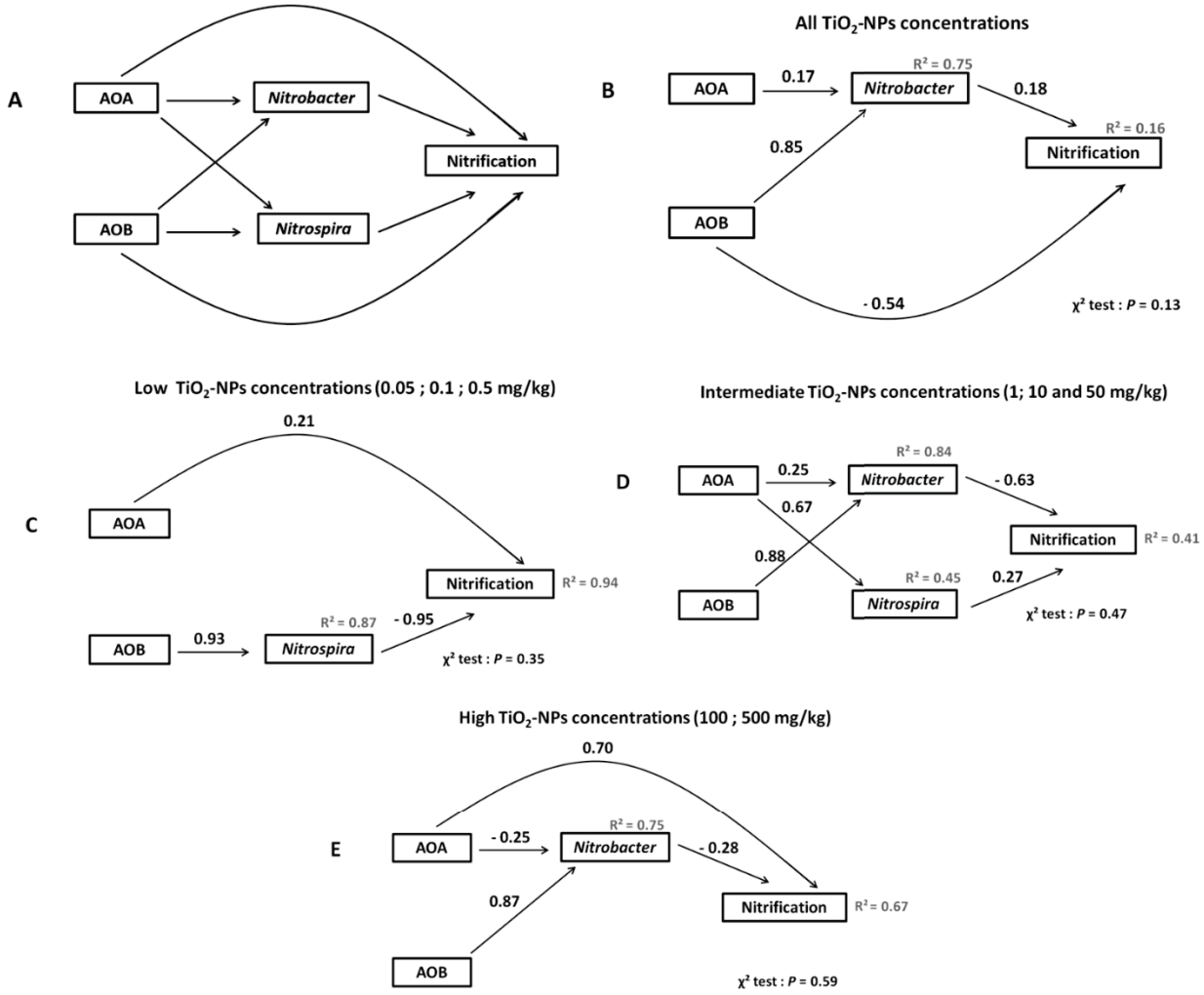


Figure 5 Path diagrams representing the theoretical full model (A) and the final models retained (B, C, D, E) to describe patterns observed in potential nitrification activity. Path diagram based on the data set with B) all TiO_2 concentrations tested, C) only the low concentrations tested (0.05; 0.1; 0.5 mg kg^{-1}), D) only the intermediate concentrations (1, 10, 50 mg kg^{-1}) and E) only the high concentrations tested (100 and 500 mg kg^{-1}). The final models were obtained when the best model-fit (χ^2 test) was achieved after removing the paths in the model in a stepwise manner. Path coefficients (values indicated next to the arrows) correspond to the standardized coefficients calculated based on the analysis of correlation matrices and indicate by how many standard deviations the effect variable would

change if the causal variable was changed by one standard deviation. The explained variation of the effects variables are given by the R^2 values (in grey).

DISCUSSION

Influence of the TiO_2-NPs concentration on aggregation and oxidative potential

TiO_2-NPs properties are modified according to the concentration present in water or in soil solution. NPs are known to immediately aggregate when added in aqueous media (Lowry et al. 2012). As already reported, we observed that the aggregation increased with the concentration in both ultrapure water and soil solution, likely due to the higher probability of particle collisions (Maximova and Dahl, 2006; Baalousha, 2009; Jeong et al., 2012). However for the concentrations below 50 mg L^{-1}, TiO_2-NPs were less aggregated in the soil solution than in ultrapure water. This is presumably due to the interactions of NPs with natural organic matter known to modify NPs surface properties and to increase steric repulsion (Baalousha, 2009; Domingos et al., 2009; Thio et al., 2011; Simonin et al., 2015a). From these results, we can assume that the NPs initially prepared in ultrapure water became less aggregated after application in soil and mixing with soil pore water, especially at the lowest concentrations.

The OP of TiO_2-NPs was also affected by their concentration. The increase of TiO_2-NPs concentration resulted in a strong decrease of their OP. These results could be related in part to the simultaneous increase of TiO_2-NPs aggregation with increasing concentration. The reactivity of NPs is generally linked to the available specific surface area of the particles (Hotze et al., 2010a; Lowry et al., 2012). The aggregation induced a decrease of the TiO_2-NPs surface area and consequently a decrease of their OP (Hotze et al., 2010b). TiO_2-NPs oxidative activity was 1.2 to 11 fold higher in soil solution than in ultrapure water and this result might be linked to their lower aggregation in soil solution. However, for the highest concentrations, the aggregation of TiO_2-NPs was similar in ultrapure water and soil solution (100 and 125 mg L^{-1}) but the oxidative activity was still higher in soil solution than in ultrapure water. These results suggest that NPs aggregation is not the only factor involved and that physicochemical properties of the soil solution might enhance the amount of ROS produced from TiO_2-NPs. In particular, metals, such as iron, present in the soil solution could

be a secondary source of ROS that depend on the dissolved organic carbon present in the solution (Gandois et al., 2010; Charrier and Anastasio, 2012).

In contrast with highly soluble pollutants (e.g. heavy metals, pesticides), TiO_2-NPs properties involved in the bioavailability and the toxicity of these emerging pollutants, such as aggregation and ROS production, are highly modified according to the concentration. This finding highlights the necessity to characterize NPs properties for each concentration used in conditions close to those encountered in soil.

Contrasted responses of ammonia-oxidizers and nitrite-oxidizers to TiO_2-NPs

The four microbial groups involved in soil nitrification responded differently to TiO_2-NPs contamination after 90 days of exposure. In this soil AOA appeared to be the most affected by TiO_2-NPs, while *Nitrospira* seemed insensitive to this contamination. AOB and *Nitrobacter* abundance exhibited similar responses illustrated by a strong positive correlation between these two groups. None of the microbial abundance measured exhibited a linear dose-response relationship in the range of the concentrations tested. Indeed, concentrations that differed by a factor of 5000 for AOA (0.1 and 500 mg kg^{-1}) or 1000 for *Nitrobacter* (0.1 and 100 mg kg^{-1}) caused a similar decrease of abundance. Furthermore, AOB abundance was reduced only at 2 intermediate concentrations (1 and 10 mg kg^{-1}). These results are not surprising as TiO_2-NPs properties are modified according to the concentration. Unlike soluble chemicals, dose-response relationships do not seem to be workable in the case of NPs in soil.

In the available literature, this is the first report of negative effects of TiO_2-NPs on soil microbial communities at such very low concentrations (< 1 mg kg^{-1}). These effects might be explained by the low aggregation and high reactivity of TiO_2-NPs likely conferring a higher toxicity and bioavailability at these low concentrations (Choi and Hu, 2009; Simon-Deckers et al., 2009; Menard et al., 2011). The effects observed at intermediate and high concentrations, despite their presumed lower reactivity and bioavailability, might be linked to a greater probability of interaction between TiO_2-NPs and soil microorganisms.

The higher sensitivity of AOA towards TiO_2-NPs was surprising as it is commonly agreed that these microorganisms are more tolerant to chronic stresses due to the specific chemical

structure of their membrane lipids which is exceptional in the microbial world (Schleper et al., 2005; Valentine, 2007). Indeed, soil AOA have been reported to be more tolerant to heavy metals than AOB (Li et al., 2009; Ollivier et al., 2012; Subrahmanyam et al., 2014), but not in all cases (Mertens et al., 2009). However, the current lack of knowledge on soil AOA physiology and ecology does not allow to propose any hypothesis for their higher sensitivity to TiO_2-NPs.

AOB and *Nitrobacter* abundance was significantly lowered at the intermediate TiO_2-NPs concentrations tested. Previous studies conducted with activated sludges, also reported a decrease of AOB and NOB (*Nitrobacter* and *Nitrospira*) abundance associated to a high reduction of ammonia- and nitrite-oxidation activities in presence of TiO_2-NPs or Ag-NPs (Liang et al., 2010; Zheng et al., 2011). However, in our study *Nitrospira* abundance was not modified whatever the concentration of TiO_2-NPs. Among soil nitrite oxidizers, *Nitrobacter* and *Nitrospira* have different ecological requirements, *Nitrobacter* being adapted to high nitrite and oxygen environments, while *Nitrospira* are adapted to low nitrite and oxygen conditions (Schramm et al., 1999; Attard et al., 2010; Wertz et al., 2012; Nowka et al., 2015; Simonin et al., 2015b). In this study, the decreases of AOB and AOA abundance and activity may have resulted in lower nitrite availability and consequently conditions favorable to *Nitrospira* compared to *Nitrobacter* (Ollivier et al., 2013).

These contrasted responses observed among ammonia- and nitrite-oxidizers according to the NPs concentrations tested suggest a different sensitivity of the four microbial groups to TiO_2-NPs and especially to their oxidative stress. Consequently, under this disturbance some modifications in the functional players of ammonia and nitrite-oxidation could be expected depending on the concentration.

Consequences on nitrification activity: a path analysis approach

As the abundance of microbial groups involved in the two steps of the nitrification was variably altered by TiO_2-NPs contamination, we used a path analysis to decipher how changes in ammonia- and nitrite-oxidizer abundance have impacted soil nitrification activity.

Interestingly, potential nitrification activity was found to be reduced only with the lowest and the highest concentrations, whereas significant decreases in nitrifier abundance were observed also in intermediate TiO_2-NPs concentrations. The path analysis encompassing all TiO_2-NPs concentrations tested did not allow to explain satisfactorily the changes in nitrification activity, probably because the links between ammonia-oxidizer abundance, nitrite-oxidizer abundance and nitrification were concentration-dependent. This is why the path analysis were further performed separately with low, intermediate and high NPs concentrations data sets. With this approach a significant part of the nitrification activity variation was explained by the models. The functional players of nitrification and the coupling between ammonia- and nitrite-oxidizers were different in the 3 path analysis (i.e low, intermediate and high concentrations). These results suggest a surprising flexibility of nitrification functioning supported by a functional redundancy among ammonia-oxidizers and nitrite-oxidizers (Wertz et al., 2007; Mertens et al., 2009). However, the significant decreases of potential nitrification induced by 3 TiO_2-NPs concentrations (0.05; 100 and 500 mg kg^{-1}) suggest that the nitrifier functional redundancy does not always allow to maintain nitrification efficiency.

In the low and high TiO_2-NPs concentrations path analyses, AOA abundance was directly linked to nitrification, suggesting their important functional role in ammonia-oxidation in this soil. Consistently, AOA abundance was on average 54 fold higher in this soil than AOB abundance. The direct path between AOA abundance and nitrification may further indicate that ammonia-oxidation can be the limiting step of nitrification as often reported (Kowalchuk and Stephen, 2001). Thus we can assume that the observed significant decreases of potential nitrification could be partially related to the decreases of AOA abundance. This assumption is supported by the positive correlation between AOA and potential nitrification (Fig. 4)

The variation of ammonia-oxidizer abundance enabled to explain a great part of NOB abundance variation (*Nitrobacter* and/or *Nitrospira* according to the TiO_2-NPs concentrations) highlighting the strong coupling between ammonia- and nitrite-oxidizers in soil (Ollivier et al., 2013; Simonin et al., 2015b). Therefore, the shifts in NOB community and the altered coupling between ammonia- and nitrite-oxidizers might also be responsible for the lower nitrification efficiency in some treatments. In particular, culture studies have

shown that AOA and *Nitrospira* have lower specific cell activity than AOB and *Nitrobacter*, respectively (Schramm et al., 1999; Prosser and Nicol, 2012; Nowka et al., 2015).

As also reported by Petersen et al. (2012), this study shows that functional gene abundance is a good predictor of potential rates that integrates recent environmental history and recent process activity. Thereby the path analysis approach permitted to understand how a disturbance altered the coupling between ammonia- and nitrite-oxidizers and the subsequent effects on potential nitrification activity. However, some variations of nitrification activity remain unexplained in the path analysis. Approaches targeting functional gene transcripts and/or DNA stable isotope probing could help identifying active nitrifiers in this soil and deciphering the contributions of AOB, AOA, *Nitrobacter* and *Nitrospira* communities to nitrification activity (Xia et al., 2011; Ruyters et al., 2013). The improvement of the path analysis could also be achieved by considering the effects of TiO_2-NPs on nitrifier diversity and community structure in order to include specific nitrifier lineages involved in nitrification and/or sensitive to TiO_2-NPs stress in the model (Cantarel et al., 2012).

Conclusions

In this soil, the nitrification was similarly reduced by a very low TiO_2-NPs concentration (0.05 mg kg^{-1}) and high concentrations (100 and 500 mg kg^{-1}). Changes in nitrification activity were explained by the alteration of the coupling between ammonia- and nitrite-oxidizers due to the different sensitivity of these microbial groups to TiO_2-NPs contamination according to the concentration.

The absence of classical dose-response relationships on nitrifier abundance was related to the modification of TiO_2-NPs properties depending on the concentration tested. TiO_2-NPs exhibited the largest toxic potential in soil solution at the lowest concentrations, due to low aggregation and high oxidative potential under these conditions. This emphasized that classical approaches for risk assessment based on dose-response toxicological tests are not pertinent for NPs in soil.

This work highlights that TiO_2-NPs could be detrimental for a key process of the N cycling and raises concern about the potential cascading effects on other essential processes for ecosystem services delivery.

ACKNOWLEDGMENTS

Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region – ARC Environnement. This work was funded by a grant from the French National Program Microbien EC2CO-CNRS. The authors thank the lab assistance of Mathilde Graillat, Sungeun Lee and Erwann Vince. Nitrification measurements were performed at the AME platform (Microbial Ecology UMR5557-USC1364, Lyon) and quantitative PCR at the DTAMB platform (IFR 41, University Lyon 1).

CONFLICT OF INTEREST

The authors declare to have no conflict of interest.

REFERENCES

Attard E, Poly F, Commeaux C, Laurent F, Terada A, Smets B, et al. Shifts between Nitrospira- and Nitrobacter-like nitrite oxidizers underly the response of soil potential nitrite oxidation to changes in tillage practices. *Environ Microbiol* 2010;**12**: 315–326.

Baalousha M. Aggregation and disaggregation of iron oxide nanoparticles: Influence of particle concentration, pH and natural organic matter. *Sci Total Environ* 2009;**407**: 2093–2101.

Brar SK, Verma M, Tyagi RD, Surampalli RY. Engineered nanoparticles in wastewater and wastewater sludge – Evidence and impacts. *Waste Manage* 2010;**30**: 504–520.

Broos K, Mertens J, Smolders E. Toxicity of heavy metals in soil assessed with various soil microbial and plant growth assays: A comparative study. *Environ Toxicol Chem* 2005;**24**: 634–640.

Cantarel AAM, Bloor JMG, Pommier T, Guillaumaud N, Moirot C, Soussana JF, Poly F. Four years of experimental climate change modifies the microbial drivers of N2O fluxes in an upland grassland ecosystem. *Glob Change Biol* 2012;**18**:2520-2531.

Cathcart R, Schwiers E, Ames BN, Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem* 1983;**134**: 111–116.

Charrier JG, Anastasio C. On dithiothreitol (DTT) as a measure of oxidative potential for ambient particles: evidence for the importance of soluble transition metals. *Atmos Chem Phys* 2012;**12**: 11317–11350.

Choi OK, Hu ZQ. Nitrification inhibition by silver nanoparticles. *Water Sci Technol* 2009;**59**: 1699.

Cornelis G, Hund-Rinke K, Kuhlbusch T, Brink N. van den Nickel C. Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. *Crit Rev Env Sci* Tec 2014;**44**: 2720–2764.

Dalzell DJB, Alte S, Aspichueta E, de la Sota A, Etxebarria J, Gutierrez M, et al. A comparison of five rapid direct toxicity assessment methods to determine toxicity of pollutants to activated sludge. *Chemosphere* 2002;**47**: 535–545.

Dassonville N, Guillaumaud N, Piola F, Meerts P, Poly F. Niche construction by the invasive Asian knotweeds (species complex Fallopia): impact on activity, abundance and community structure of denitrifiers and nitrifiers. *Biol Invasions* 2011;**13**: 1115–1133.

Domingos RF, Tufenkji N, Wilkinson KJ Aggregation of Titanium Dioxide Nanoparticles: Role of a Fulvic Acid. *Environ. Sci. Technol.* 2009;**43**: 1282–1286.

Editorial Nature Nanotechnology. The dose makes the poison. *Nature Nanotech* 2011;**6**:329-329

Freitag T, Chang L, Clegg C, Prosser J. Influence of inorganic nitrogen management regime on the diversity of Nitrite-Oxidizing Bacteria in agricultural grassland soils. *Appl Environ Microbiol* 2005;**71**: 8323–8334.

French RA, Jacobson AR, Kim B, Isley SL, Penn RL, Baveye PC. Influence of Ionic Strength, pH, and Cation Valence on Aggregation Kinetics of Titanium Dioxide Nanoparticles. *Environ Sci Technol* 2009;**43**: 1354–1359.

Gandois L, Probst A, Dumat C. Modelling trace metal extractability and solubility in French forest soils by using soil properties. *Eur J Soil Sci* 2010;**61**: 271–286.

Gelfand I, Yakir D. Influence of nitrite accumulation in association with seasonal patterns and mineralization of soil nitrogen in a semi-arid pine forest. *Soil Biol Biochem* 2008;**40**: 415–424.

Ge Y, Schimel J, Holden P. Evidence for negative effects of TiO2 and ZnO nanoparticles on soil bacterial communities. *Environ Sci Technol* 2011;**45**: 1659–1664.

Hotze EM, Bottero JY, Wiesner MR Theoretical framework for nanoparticle reactivity as a function of aggregation state. *Langmuir* 2010a;**26**: 11170–11175.

Hotze EM, Phenrat T, Lowry GV. Nanoparticle Aggregation: Challenges to Understanding Transport and Reactivity in the Environment. *J Environ Qual* 2010b;**39**: 1909.

Jeong E, Chae SR, Kang ST, Shin HS. Effects of silver nanoparticles on biological nitrogen removal processes. *Water Sci Tech* 2012;**65**: 1298–1303.

Keller AA, McFerran S, Lazareva A, Suh S. Global life cycle releases of engineered nanomaterials. *J Nanopart Res* 2013;**15**: 1–17.

Keller AA, Wang H, Zhou D, Lenihan HS, Cherr G, Cardinale BJ, et al. Stability and Aggregation of Metal Oxide Nanoparticles in Natural Aqueous Matrices. *Environ Sci Technol* 2010;**44**: 1962–1967.

Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, et al. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environ Toxicol Chem* 2008;**27**: 1825–1851.

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 2005;**437**: 543–546.

Kowalchuk G, Stephen J. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu RevMicrobiol* 2001;**55**: 485–529.

Leininger S, Urich T, Schloter M et al. Archaea predominate among ammonia-oxydizing prokaryotes in soils. *Nature* 2006;**442**: 806–809.

Liang Z, Das A, Hu Z. Bacterial response to a shock load of nanosilver in an activated sludge treatment system. *Water Res* 2010;**44**: 5432–5438.

Li X, Zhu Y-G, Cavagnaro TR, Chen M, Sun J, Chen X, Qiao M. Do ammonia-oxidizing archaea respond to soil Cu contamination similarly as ammonia-oxidizing bacteria? *Plant Soil* 2009;**324**: 209–217.

Lowry GV, Gregory KB, Apte SC, Lead JR. Transformations of Nanomaterials in the Environment. *Environ Sci Technol* 2012;**46**: 6893–6899.

Maximova N, Dahl O. Environmental implications of aggregation phenomena: Current understanding. *Curr Opin Colloid In* 2006;**11**: 246–266.

Menard A, Drobne D, Jemec A. Ecotoxicity of nanosized TiO2. Review of in vivo data. *Environ Pollut* 2011;**159**: 677–684.

Mertens J, Broos K, Wakelin SA, Kowalchuk GA, Springael D, Smolders E. Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. *ISME J* 2009;**3**: 916–923.

Mitrano DM, Motellier S, Clavaguera S, Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. *Environment Int* 2015;**77**: 132–147.

Neal AL. What can be inferred from bacterium–nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? *Ecotoxicology* 2008;**17**: 362–371.

Nowka B, Daims H, Spieck E. Comparison of Oxidation Kinetics of Nitrite-Oxidizing Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation. *Appl Environ Microbiol* 2015;**81**: 745–753.

Ollivier J, Schacht D, Kindler R, Groeneweg J, Engel M, Wilke B-M. et al. Effects of repeated application of sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions. *Front Microbiol* 2013;**4**, doi: 10.3389/fmicb.2013.00022

Ollivier J, Wanat N, Austruy A, Hitmi A, Joussein E, Welzl G, et al. Abundance and Diversity of Ammonia-Oxidizing Prokaryotes in the Root–Rhizosphere Complex of Miscanthus × giganteus Grown in Heavy Metal-Contaminated Soils. *Microb Ecol* 2012;**64**: 1038–1046.

Pereira e Silva MC, Poly F, Guillaumaud N, van Elsas JD, Falcão Salles J. Fluctuations in ammonia oxidizing communities across agricultural soils are driven by soil structure and pH. *Front Microbiol* 2012;**3**, doi: 10.3389/fmicb.2012.00077

Petersen DG, Blazewicz SJ, Firestone M, Herman DJ, Turetsky M, Waldrop M. Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. *Environ Microbiol* 2012;**14**: 993–1008.

Pokhrel LR, Dubey B, Scheuerman PR. Natural water chemistry (dissolved organic carbon, pH, and hardness) modulates colloidal stability, dissolution, and antimicrobial activity of citrate functionalized silver nanoparticles. *Environ. Sci.: Nano* 2014;**1**: 45–54.

Prosser JI, Nicol GW. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends Microbiol* 2012;**20**: 523–531.

R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Roux-Michollet D, Czarnes S, Adam B, Berry D, Commeaux C, Guillaumaud N, et al. Effects of steam disinfestation on community structure, abundance and activity of heterotrophic, denitrifying and nitrifying bacteria in an organic farming soil. *Soil Biol Biochem* 2008;**40**: 1836–1845.

Ruyters S, Mertens J, Springael D, Smolders E. Stimulated activity of the soil nitrifying community accelerates community adaptation to Zn stress. *Soil Biol Biochem* 2010;**42:** 766-772.

Ruyters S, Nicol GW, Prosser JI, Lievens B, Smolders E. Activity of the ammonia oxidising bacteria is responsible for zinc tolerance development of the ammonia oxidising community in soil: a stable isotope probing study. *Soil Biol Biochem* 2013;**58**: 244-247.

Schleper C, Jurgens G, Jonuscheit M. Genomic studies of uncultivated archaea. *Nat Rev Micro* 2005;**3**: 479–488.

Schramm A, Beer D, de Heuvel JC, van den Ottengraf S, Amann R. Microscale Distribution of Populations and Activities of Nitrosospira and Nitrospira spp. along a Macroscale Gradient in a Nitrifying Bioreactor: Quantification by In Situ Hybridization and the Use of Microsensors. *Appl Environ Microbiol* 1999;**65**: 3690–3696.

Servin A, Elmer W, Mukherjee A, De La Torre-Roche R, Hamdi H, White JC, et al. A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. *J Nanopart Res* 2015;**17**: 1–21.

Shipley B. Cause and Correlation in Biology: A User's Guide to Path Analysis, Structural Equations and Causal Inference Cambridge University Press, 2002.

Simon-Deckers A, Loo S, Mayne-L'hermite M, Herlin-Boime N, Menguy N, Reynaud C, et al. Size-, Composition- and Shape-Dependent Toxicological Impact of Metal Oxide Nanoparticles and Carbon Nanotubes toward Bacteria. *Environ Sci Techno.* 2009;**43**: 8423–8429.

Simonin M, Guyonnet JP, Martins JMF, Ginot M, Richaume A. Influence of soil properties on the toxicity of TiO2 nanoparticles on carbon mineralization and bacterial abundance. *J Hazard Mater* 2015a;**283**: 529–535.

Simonin M, Le Roux X, Poly F, Lerondelle C, Hungate BA, Nunan N, Niboyet A. Coupling Between and Among Ammonia Oxidizers and Nitrite Oxidizers in Grassland Mesocosms Submitted to Elevated CO2 and Nitrogen Supply. *Microb Ecol,* 2015b; doi 10.1007/s00248-015-0604-9.

Simonin, M, Richaume A. Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review. *Environ Sci Pollut Res,* 2015; doi 10.1007/s11356-015-4171-x.

Subrahmanyam G, Hu H-W, Zheng Y-M, Gattupalli A, He J-Z, Liu Y-R. Response of ammonia oxidizing microbes to the stresses of arsenic and copper in two acidic alfisols. *Appl Soil Ecol* 2014;**77**: 59–67.

Sun TY, Gottschalk F, Hungerbühler K, Nowack B. Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environ Pollut* 2014;**185**: 69–76.

Thio BJR, Zhou D, Keller AA. Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles. *J Hazard Mater* 2011;**189**: 556–563.

Tourinho PS, van Gestel CAM, Lofts S, Svendsen C, Soares AMVM, Loureiro S. Metal-based nanoparticles in soil: Fate, behavior, and effects on soil invertebrates. *Environ Toxicol Chem* 2012;**31**: 1679–1692.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk H-P, Schleper C. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* 2005;**7**: 1985–1995.

Valentine DL. Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat Rev Micro* 2007;**5**: 316–323.

Vitorge E, Szenknect S, Martins JMF, Barthès V, Auger A, Renard O, Gaudet J-P. Comparison of three labeled silica nanoparticles used as tracers in transport experiments in porous media. Part I: Syntheses and characterizations. *Environ Pollut* 2014;**184**: 605–612.

Vitorge E, Szenknect S, Martins JMF, Gaudet J-P. Size- and concentration-dependent deposition of fluorescent silica colloids in saturated sand columns: transport experiments and modeling. *Environ. Sci.: Processes Impacts* 2013;**15**: 1590–1600.

Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Guillaumaud N, Le Roux X. Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. *Environ Microbiol* 2007;**9**: 2211–2219.

Wertz S, Leigh AKK, Grayston SJ. Effects of long-term fertilization of forest soils on potential nitrification and on the abundance and community structure of ammonia oxidizers and nitrite oxidizers. *FEMS Microbiol Ecol* 2012;**79**: 142–154.

Wessén E, Hallin S. Abundance of archaeal and bacterial ammonia oxidizers – Possible bioindicator for soil monitoring. *Ecological Indicators* 2011;**11**: 1696–1698.

Xia T, Kovochich M, Liong M, Mädler L, Gilbert B, Shi H, et al. Comparison of the Mechanism of Toxicity of Zinc Oxide and Cerium Oxide Nanoparticles Based on Dissolution and Oxidative Stress Properties. *ACS Nano* 2008;**2**: 2121–2134.

Xia W, Zhang C, Zeng X, Feng Y, Weng J, Lin X, et al. Autotrophic growth of nitrifying community in an agricultural soil. *ISME J* 2011.**5**: 1226–1236.

Zhalnina K, de Quadros PD, Camargo FAO, Triplett EW. Drivers of archaeal ammonia-oxidizing communities in soil. *Front Microbiol* 2012;**3**, doi: 10.3389/fmicb.2012.00210

Zheng X, Chen Y, Wu R. Long-Term Effects of Titanium Dioxide Nanoparticles on Nitrogen and Phosphorus Removal from Wastewater and Bacterial Community Shift in Activated Sludge. *Environ Sci Technol* 2011;**45**: 7284–7290.

4. Comparaison des effets de contaminations aigüe et chronique sur le transport et la toxicité du TiO_2 sur la nitrification

a. Article 6 : Présentation générale de l'étude et synthèse des principaux résultats

Nos travaux ont permis de montrer que la toxicité des NPs peut s'exprimer après une durée de trois mois dans des microcosmes de sols et que les communautés microbiennes affectées sont peu résilientes à cette perturbation. Il s'agissait, dans toutes les expériences, de contaminations aigües aux TiO_2-NPs (i.e. 1 seule contamination), or il est plus vraisemblable que les sols soient exposés à ces contaminants de manière chronique à court ou long terme. Par exemple, l'irrigation ou l'utilisation de nanofertilisants ou nanopesticides peuvent être renouvelés plusieurs fois la même année entrainant une exposition chronique des sols agricoles. L'absence de résilience et les effets apparaissant à long terme dans nos précédentes expérimentations suscitent des interrogations quant à la réponse des microorganismes exposés de façon chronique aux TiO_2-NPs. En effet, on peut faire l'hypothèse que les effets observés sur des communautés microbiennes déjà exposées à cette perturbation mais non résistantes sera amplifié lorsqu'une nouvelle contamination aura lieu. Il est également possible que des effets négatifs n'apparaissent qu'après un apport chronique. Ces aspects n'ayant pas fait l'objet d'études répertoriées dans la littérature, il nous a paru nécessaire de mettre en place des dispositifs permettant d'étudier à la fois les conséquences de contaminations aigüe et chronique sur les microorganismes du sol. L'utilisation de microcosmes de sol ne permet pas de réaliser des apports multiples de NPs, lorsqu'elles sont apportées en suspension, sans modifier le statut hydrique du milieu. Par ailleurs, les microcosmes de sol constituent un système statique dans lequel les transferts verticaux sont difficilement pris en compte.

Dans des situations réalistes de contamination du sol par les NPs, ces dernières seront apportées en surface et pourront être entrainées en profondeur par les mouvements d'eau, par exemple suite à un arrosage ou une pluie. Dans ces conditions, on peut penser que la distribution des NPs dans les sols sera hétérogène et on peut s'attendre à un gradient de concentration du polluant en fonction de la profondeur, selon leurs capacités de transport dans le sol considéré. L'absence de relation dose-réponse linéaire associée aux TiO_2-NPs rend difficile la prédiction de l'effet d'une contamination ayant une distribution hétérogène

dans le sol, car de fortes concentrations peuvent avoir des effets similaires à ceux de faibles concentrations (Article 5).

Nous proposons donc d'utiliser un système dynamique basé sur des colonnes de sol comme nous l'avions utilisé pour les expériences de transport (Article 2), afin d'apprécier les effets d'une exposition plus réaliste par un apport en surface. Ce dispositif permet de réaliser à la fois des contaminations aigües et chroniques et d'étudier en parallèle le transport du contaminant et son impact sur les communautés microbiennes à différentes profondeurs du sol. Dans notre expérimentation, nous avons réalisé 3 scénarios d'exposition permettant d'obtenir une même concentration totale finale en TiO_2-NPs (372 mg kg^{-1}) apportée dans le sol limono-argileux :

- Scénario 1 : Contamination aigüe= 1 apport de TiO_2-NPs à 50 mg L^{-1}
- Scénario 2 : Contamination chronique= 2 apports de TiO_2-NPs à 25 mg L^{-1}
- Scénario 3 : Contamination chronique= 3 apports de TiO_2-NPs à 16.7 mg L^{-1}

Pour les colonnes recevant plusieurs apports de TiO_2-NPs, les différentes contaminations ont été réalisées avec un intervalle de 15 jours d'incubation entre chaque. Durant chaque apport, les effluents des colonnes ont été collectés afin d'étudier le transport du TiO_2.

Pour chacun de ces scénarios, après la dernière exposition, le sol des colonnes a été récupéré, découpé en différentes couches en fonction de la profondeur (0-2 cm ; 2-4 cm ; 4-6 cm ; 6-8 cm) puis incubé dans des microcosmes pendant 60 jours afin d'évaluer l'impact des NPs sur les communautés microbiennes (Figure 17). L'indicateur écologique choisis a été la nitrification en mesurant les effets sur l'activité nitrifiante et l'abondance des AOA et AOB.

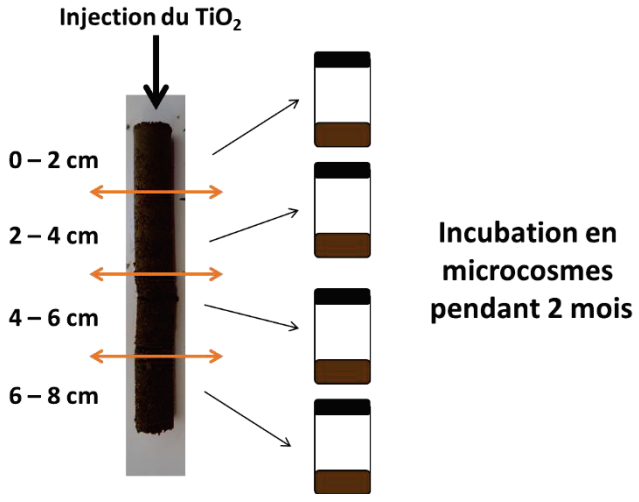


Figure 17: Découpage d'une colonne de sol en 4 profondeurs avant son incubation en microcosmes.

En accord avec l'article 2, nos résultats montrent que le transport du TiO_2 est très faible et en plus qu'il dépend de la concentration appliquée lors d'une contamination aigüe (i.e. 1er apport). La mobilité du TiO_2 augmente lorsque la concentration utilisée diminue (bilan de masse TiO_2 : 50 mg L^{-1} = 2.2% ; 25 mg L^{-1} = 5% ; 16.7 mg L^{-1} = 11%). Cela peut être expliqué par le fait que l'agrégation des TiO_2-NPs diminue aux plus faibles concentrations, ce qui favorise leur transport dans la porosité du sol. Lors de la seconde contamination, nous avons constaté que la mobilité du TiO_2 était fortement réduite comparée au premier apport, quelle que soit la concentration testée (25 et 16.7 mg L^{-1}). Pour les colonnes recevant un 3ème apport, le transport du polluant était négligeable car 99.8% du TiO_2 amené était retenu dans le sol. La concentration finale retenue dans le sol a été calculée à partir des bilans de masse obtenus. Nous avons observé que la concentration en TiO_2 la plus forte était observée dans les colonnes soumises à une contamination aigüe (364 mg kg^{-1}) et la plus faible dans celles exposées 3 fois aux TiO_2-NPs (328 mg kg^{-1}).

Concernant l'évaluation de l'impact sur la nitrification dans les 3 scénarios d'exposition, aucun effet négatif n'a été observé dans les sols soumis à 1 ou 2 apports de TiO_2-NPs. En revanche, une diminution significative de l'activité nitrifiante et de l'abondance des AOA et des AOB a été constatée après 3 apports de TiO_2-NPs sur l'ensemble de la colonne (i.e. moyenne des 4 profondeurs). Un effet significatif de la profondeur a été uniquement observé sur l'activité nitrifiante pour laquelle une diminution plus importante a été notée dans les 2 premiers centimètres du sol, zone probable d'accumulation préférentielle de NPs comme le prédit la théorie de la filtration des colloïdes (e.g. Vitorge 2010, Vitorge *et al.,* 2014).

En conclusion, ces travaux montrent qu'il est fondamental de comparer les effets de contaminations aigües et chroniques en conditions réalistes d'exposition. En effet dans ces conditions simulant un apport en surface, une contamination aigüe à forte concentration n'a pas eu d'effet alors que 3 contaminations résultant à une concentration 10% plus faible ont eu des effets délétères sur la nitrification. Il serait nécessaire de considérer la résistance et la résilience des communautés microbiennes lors d'expositions chroniques en étudiant les effets après chaque exposition et avant la suivante pour comprendre et prédire l'effet de contaminations répétées.

Dans cette expérimentation, nous avons également mis en évidence qu'à partir de la seconde exposition, les TiO_2-NPs étaient peu mobiles dans le sol, sans doute suite au

colmatage partiel des pores situés dans les premiers centimètres du sol, qui ne permettent alors plus le transport de ces contaminants. Toutefois, pour le vérifier nous n'avons pas pu mesurer la concentration en TiO_2-NPs aux différentes profondeurs du sol en raison de la très forte concentration naturelle du sol en titane (4.52 g kg^{-1}) comparée aux concentrations apportées.

b. « Transport of TiO_2 nanoparticles and toxicity on microbial communities under acute and chronic exposures in soil columns»

L'article 6 intitulé «Transport of TiO_2 nanoparticles and toxicity on microbial communities under acute and chronic exposures in soil columns» est en préparation en vue d'une soumission dans la revue *Environmental Science & Technology*.

Transport of TiO_2 nanoparticles and toxicity on microbial communities under acute and chronic exposures in soil columns

Marie SIMONIN[1,2,3,4], Jean M. F. MARTINS[4], Gaëlle UZU[4], Erwann VINCE[4] and Agnès RICHAUME[1,2,3*]

[1] Université de Lyon, Lyon, France

[2] Université Claude Bernard Lyon 1, Villeurbanne, France

[3] CNRS, UMR 5557, Ecologie Microbienne, Université Lyon 1, Villeurbanne, France

[4] UJF-Grenoble/CNRS-INSU/G-INP/IRD, LTHE UMR 5564, Grenoble F-38041, France

[*]Correspondence to: agnes.richaume@univ-lyon1.fr

ABSTRACT

Soils are being extensively exposed to nanoparticles (NPs) due to their increasing use in many commercial products. The adverse effect of NPs on soil microorganisms has been reported in several ecotoxicological studies with various experimental setups, such as microcosms or field lysimeters. Most of these studies were based on acute exposures to NPs although chronic exposures are more likely to occur. Contrarily to single acute contaminations, the evaluation of soil exposure to chronic NPs contaminations requires the use of specific setups. Using a soil column approach, we compared the influence of acute and chronic contaminations on the transport of TiO_2-NPs through soil. The effect of these different exposure scenarios on the abundance and activity of soil nitrifying microbial community after 2 months of incubation was evaluated. Soil columns were contaminated using 3 exposure scenarios (1, 2 or 3 TiO_2-NPs applications) resulting in the same TiO_2-NPs final concentration applied in columns of a silty-clay soil. The transport of TiO_2-NPs was very low in this soil under both acute and chronic exposures and was influenced by NPs injection concentration during the first application of each scenario. TiO_2-NPs mobility increased at lower concentration wherein TiO_2-NPs were less aggregated in the soil solution compared to the highest concentration. Significant decreases of nitrification activity and ammonia-oxidizing archaeal and bacterial abundances were observed only in chronic exposure condition, in the 3 TiO_2-NPs applications scenario.

Thus, this study highlights that under chronic exposure, the transport of TiO_2-NPs to deeper soil layers and groundwater is likely limited and that a chronic contamination was more damaging for the microbiological functioning than an acute exposure. This work suggests that more studies on the fate and impact of chronic NPs contaminations are clearly needed as they appear, contrarily to acute exposure, as the more favourable situations for NPs impact in soil

Key-words: TiO_2 Nanomaterials, Chronic and acute exposure, transport, Microbial Ecotoxicology, Nitrification.

INTRODUCTION

In recent years, a significant number of studies highlighted deleterious effects of metal-oxide nanoparticles (NPs) on soil (micro)organisms and consequently on soil functioning and fertility (Dinesh *et al.*, 2012; Pan and Xing, 2012; Simonin and Richaume, 2015). In most of these studies, soils were exposed to acute homogeneous NPs contaminations in microcosms (Simonin and Richaume, 2015). However, NPs are more likely to be released on soil surface through repeated applications of sewage sludge, irrigation or the use of nanofertilizers and nanopesticides (Brar *et al.*, 2010; Liu and Lal, 2015; Servin *et al.*, 2015) and are then leached in depth into soil porosity (Cornelis *et al.*, 2014). Thereby in natural soils, NPs are heterogeneously distributed, especially along the vertical soil profile (Navarro *et al.*, 2011). This heterogeneous distribution of NPs is largely controlled by the soil physicochemical properties that influence their mobility in agreement with the colloidal filtration theory (Fang *et al.*, 2009).

Furthermore although soils are mostly chronically exposed to NPs, most transport and ecotoxicological studies generally consider a single and high NPs concentration as a chronic exposure. To date, only few studies have actually performed experiments with repeated soil exposures to NPs (Hooper *et al.*, 2011). The lack of such studies and consequently of knowledge on NPs chronic toxicity in soil is mainly due to the difficulty to perform chronic exposures in microcosm experiments. In microcosms, soil moisture must be kept constant all along the soil incubation and repeated aqueous applications of NPs for simulating chronic exposures would induce moisture stresses in addition to the NPs toxicity, thus limiting the validity of such approaches. Therefore, several aspects remain to be investigated to understand the fate and toxicity of NPs. Specifically, it is necessary to use more realistic experimental conditions of contamination simulating chronic exposure with the application of NPs on soil surface, in order to consider their heterogeneous distribution according to soil depth.

The aim of this work was to compare the effect of acute and chronic contaminations on (i) the transport of titanium dioxide NPs (TiO_2-NPs) through a soil profile and (ii) their toxicity on soil microbial community by focusing on the key microbial process of nitrification involved in the nitrogen cycle. TiO_2-NPs were chosen because they are the most produced

NPs and it is predicted that they would represent 50% of all NPs retrieved in soil (Keller *et al.*, 2013; Sun *et al.*, 2014).

The experimental design was based on soil columns and enabled the study of the fate and impact of both acute and chronic TiO_2-NPs exposures in soil under more realistic conditions of exposure than those usually carried out using microcosms. Soil columns were contaminated using 3 exposure scenarios (1, 2 or 3 contaminations) resulting in the same total TiO_2-NPs concentrations applied in soil columns (372 mg NPs kg^{-1} dry soil). After the last NPs application, four layers of the contaminated soil columns corresponding to different depths were incubated separately into glass flasks for 2 months to study the NPs impact on the nitrification process. The potential nitrification activity and the abundance of ammonia-oxidizing archaea and bacteria (AOA and AOB respectively) involved in the first step of nitrification were measured and interpreted in the light of NPs physicochemical properties and mobility in soil.

MATERIALS AND METHODS

Soil

The upper 20 cm layer of a silty-clay soil (Cambisol, WRB, 2006) was collected in Commarin (Côte d'Or, France) under a permanent pasture. After collection, roots and plant litter were manually removed. The soil was sieved at 2 mm and homogenized before storage at 4°C. The main soil characteristics were: sand 10%; loam 51%; clay 39%; pH 7.7; OM 7.9%; CEC 20.1 cmol kg^{-1}; water holding capacity (WHC) 51%; ionic strength 1.37 mM.

TiO_2 Nanoparticles

Titanium dioxide nanoparticles (TiO_2-NPs) were provided by Sigma Aldrich (St Louis, USA) (mixture of anatase (80%) and rutile (20%) crystal structure) with at least 99.5% purity. TiO_2-NPs mean particle size was 28.7 nm in powder as measured with a ZEISS Ultra 55 scanning electron microscopy–field emission gun (SEM-FEG) and energy dispersive spectroscopy (EDS) with a SDD detector (BRUKERAXS-30 mm^2).

The aggregated size and zeta potential of the TiO_2-NPs were characterized using Dynamic Light Scattering (DLS) in the soil solution used as background solution for the column experiments. Soil solution was prepared following the protocol described by Simonin *et al.* (2015). The measurements were performed in the different suspensions of TiO_2-NPs (50, 25 and 16.7 mg L^{-1}), after dispersion by ultrasonication for 5 minutes to ensure suspensions homogeneity. For each sample, the mean of 3 measurements was recorded.

Exposure of soil columns to acute and chronic applications of TiO_2-NPs

Three TiO_2-NPs suspensions were prepared in soil solution in order to obtain a final total concentration of 372 mg kg^{-1} dry soil in the soil columns, whatever the number of exposures with 50, 25 and 16.7 mg L^{-1} for 1, 2 and 3 exposures, respectively.

The soil exposures to NPs were performed in 1 x 10 cm small glass columns (C10/10, GE Healthcare), homogeneously packed with 8 cm of wet soil, at 28°C in the dark. Flow adaptors (AC 10, GE Healthcare) were adjusted on the top of the columns to ensure constant and similar soil heights during experiments. At the beginning of the experiment, the columns were saturated and leached bottom-up with 100 mL of soil solution. Then, the background solution was replaced by the TiO_2-NPs suspension that was injected for 5 pore volumes (PV) (i.e. 6.7 ml) at a flow rate of 0.1 mL min^{-1}. Then, 10 PV of the soil solution free of TiO_2-NPs were injected.

The acute exposure experiment consisted in the injection of 5 PV of 50 mg L^{-1} TiO_2-NPs into 6 saturated soil columns. Six control columns received the same PV of soil solution free of TiO_2-NPs.

Chronic exposures experiments consisted in two or three applications of TiO_2-NPs (25 and 16.7 mg L^{-1} respectively) in 6 columns for each treatment. Six additional control columns were realized for each type of exposure. The different applications were separated by a 15 day delay during which columns were maintained horizontally at 28°C in the dark at constant moisture.

Transport of TiO_2-NPs

In each exposure treatment, the effluents from a randomly chosen column were sampled in 15 mL centrifuge tubes every 10 minutes (~1 mL) using a fraction collector (Gilson, Minipulse 3). In order to establish the TiO_2 breakthrough curves, titanium concentrations in the spiking suspensions (C0) and in the effluents (C) were determined (C/C0) using a microwave assisted (Novawave, SCP Science) strong acid extraction (hydrofluoric acid + nitric acid). Titanium concentrations were measured by inductively coupled plasma - optical emission spectrophotometer (ICP-OES; Varian 700-ES, Varian Inc. Scientific Instruments, Palo Alto, USA). Control columns leached with NPs-free soil solution enabled to determine the background concentration of titanium in the effluents. In order to ensure the quality of our titanium measurements, a certified reference material was measured along with our samples.

Consistently with Nickel *et al.,* (2015), the TiO_2-NPs concentration retained in the different soil layers after contamination could not be measured due to the high titanium content of the soil (4.52 g kg^{-1}) and the low TiO_2-NPs input used (372 mg kg^{-1}) compared to this background content.

Impact of TiO_2-NPs on nitrification process according to the soil depth

The impact of TiO_2-NPs on nitrification and ammonia-oxidizer abundance in function of soil depth was studied after the different exposure scenarios. After the last TiO_2-NPs exposure, soil columns were cut into four layers (0–2 cm, 2–4 cm, 4–6 cm and 6–8 cm) according to the depth from the inlet and placed into 60 ml glass flasks. This experimental design resulted in 144 microcosms incubated for 2 months at 28°C in the dark at constant moisture: 6 types of column treatments (3 exposure scenarios + 3 controls) x 4 depths x 6 replicates. At the end of the incubation time, 1.5 g of soil (eq dw) were immediately used for the measurements of potential nitrification and 0.5 g of soil were stored at -20°C before DNA extraction.

DNA extraction and quantification of ammonia-oxidizer abundance

DNA was extracted from 0.5 g of frozen soil using the Power Soil™ DNA Isolation Kit (MO BIO laboratories, Carlsbad, CA, USA), following the manufacturer's instructions and then DNA concentrations were determined using the Qubit dsDNA BR Assay (Invitrogen).

The abundance of the ammonia-oxidizers (AOA and AOB) was measured by quantitative PCR using a Lightcycler 480 (Roche Diagnostics, Meylan, France) and the primers and thermal cycling conditions used are described in Table 1.

The *amoA* AOA and AOB quantification were performed in a final reaction volume of 20 µL and contained (final concentrations) 0.5 µM of each primer for the bacterial *amoA* or 0.75µM of CrenamoA616r and 1µM of CrenamoA23f for the archaeal *amoA*, 2% bovine serum albumin (BSA), 1X of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 10 ng of soil DNA extract or 10^7- 10^2 gene copies number of an in-house plasmid containing cloned bacterial (*Nitrosomonas europaea*, GenBank accession number:L08050) and archaeal (54d9 fosmide fragment, (Treusch *et al.*, 2005)) *amoA* genes. Melting curves analysis confirmed the specificity of amplification of the four genes.

Table 1 PCR primers and thermal cycling conditions used for quantification of ammonia- and nitrite-oxidizer abundances

Primers	Target gene	Reference	Thermal conditions
CrenamoA23f	AOA *amoA*	Tourna *et al.*, 2008	15 min at 95°C, 45 cycles (45 s at 94°C,
CrenamoA616r	AOA *amoA*	Tourna *et al.*, 2008	45 s at 55°C and 45 s at 72°C)
amoA-1F	AOB *amoA*	Rotthauwe *et al.*, 1997	15 min at 95°C, 45 cycles (30 s at 95°C,
amoA-2R	AOB *amoA*	Rotthauwe *et al.*, 1997	45 s at 54°C, 45 s at 72°C and 15 s at 80°C)

Nitrification activity

Potential nitrification activity was determined according to the protocol described by Dassonville *et al.,* (2011). Sub-samples of fresh soil (1.5 g equivalent dry soil) were incubated with 3 ml of a solution of $(NH_4)_2SO_4$ in order to reach 50 µg N-NH_4^+ g^{-1} dry soil. Distilled water was added in each sample to achieve 12 ml of total liquid volume in plasma flasks. The flasks were sealed with Parafilm® and incubated at 28°C under 180 rpm constant agitation. During

the incubation, 1 ml of soil slurry was sampled at 2h, 4h, 6h, 8h and 10h, filtered (0.2 µm pore size) and transferred in vials stored at -20°C. The analysis of NO_3^- concentrations was performed by ionic chromatography (DX120, Dionex, Salt Lake City, USA) equipped with a 4 mm×250 mm column (IonPac AS9 HC). Potential nitrification was expressed as µg N-NO_3- $h^{-1}g^{-1}$ dry soil.

Statistical analysis

All results are presented as means (± standard error). An analysis of variance (ANOVA) and *post-hoc* Tukey HSD were performed to test the effect of TiO_2-NPs exposures and soil depth on the potential nitrification and ammonia-oxidizer abundance for each type of exposure scenario separately. Data were log-transformed prior to analysis when necessary, to ensure conformity with the assumptions of normality and homogeneity of variances. T-tests were conducted to compare the aggregation and zeta potential of TiO_2-NPs in the different spiking suspensions. All statistical analyses were carried out using R statistical software 2.13.2 (R Core Team, 2015).

RESULTS

Characteristics of TiO_2-NPs in the spiking soil solutions

TiO_2-NPs were characterized in the different suspensions used to contaminate the soil columns for the 3 exposure scenarios. The aggregation of TiO_2-NPs increased with concentration (Table 2). In the lowest concentration applied 3 times in the soil column (16.7 mg L^{-1}), TiO_2-NPs hydrodynamic diameter was 111.2 nm in average, while in the highest concentration used for the single exposure (50 mg L^{-1}), TiO_2-NPs hydrodynamic diameter was 153.9 nm.

Surface charge of TiO_2-NPs assessed through zeta potential values were relatively close in the 3 spiking suspensions (-15.4 to -16.6 mV; Table 2), even if it was significantly lower in the 25 mg L^{-1} suspension.

Table 2 Size and zeta potential of TiO_2-NPs in soil solution for the 3 tested concentrations. Means and standard errors are presented (n=3). Values labeled with the same letter do not differ at $P < 0.05$.

TiO_2 injection concentration	Hydrodynamic diameter (nm)	Zeta potential (mV)
50 mg L^{-1}	153.9 ± 10 b	-16.6 ± 0.2 a
25 mg L^{-1}	120.8 ± 18 ab	-15.4 ± 0.1 b
16.7 mg L^{-1}	111.2 ± 10 a	-16.4 ± 0.3 ab

Effect of the concentration and the number of applications on TiO_2-NPs mobility in soil

The experimental design enabled to study the influence of TiO_2-NPs concentration on their transport through soil after acute contaminations at 16.7, 25 or 50 mg L^{-1} (first application) and after chronic exposures consisting in 2 or 3 NPs applications (Table 3).

The mobility of TiO_2-NPs decreased when increasing TiO_2-NPs concentration (Table 3). At an injection concentration of 16.7 mg L^{-1}, 10.66% of injected TiO_2-NPs were recovered in the effluents, whereas only 4.95 and 2.19% were recovered at 25 and 50 mg L^{-1}, respectively.

In the columns receiving two applications, the mobility of TiO_2-NPs was greatly reduced compared to the 1st application (Table 3). Only 0.9% of the added TiO_2-NPs were recovered in the column effluents for both concentrations (Table 3). For the 3rd NPs application at 16.7 mg L^{-1}, the mobility of TiO_2-NPs was almost negligible (0.2%; Table 3).

We calculated the final TiO_2-NPs concentration retained in the column using the relative mass recovery of TiO_2-NPs in the effluents (Table 3). The same theoretical TiO_2-NPs concentration was applied (372 mg kg^{-1}) to the soil columns but the final TiO_2-NPs concentration differed in the 3 exposure scenarios (Table 3). The final highest concentration of TiO_2-NPs was found in the columns submitted to the acute exposure (364 mg kg^{-1}) with 50 mg L^{-1}. In the columns receiving 2 NPs applications at 25 mg L^{-1}, the final TiO_2-NPs concentration was estimated as 350 mg kg^{-1}, which corresponds to a decrease of 6% compared to the acute exposure. After 3 applications at 16.7 mg L^{-1}, only 328 mg kg^{-1} of

TiO_2-NPs were retained in soil, which corresponds to a decrease of 12% and 6%, compared to the 1 and 2 exposure scenarios, respectively.

Table 3 TiO_2 relative mass recovery in the column effluents for the three applied concentrations in the different exposure scenarios (1, 2 or 3 applications). Final TiO_2-NPs concentration retained in the column is calculated from the relative mass recovery of TiO_2-NPs in the effluents.

TiO_2 injection concentration	Relative mass recovery of TiO_2-NPs:			Final TiO_2 concentration in soil (mg kg^{-1} dry soil)
	1st application	2nd application	3rd application	
50 mg L^{-1}	2.19%			363.9
25 mg L^{-1}	4.95%	0.94%		350.1
16.7 mg L^{-1}	10.66%	0.93%	0.21%	328.1

Effect of acute and chronic contaminations on nitrification activity and ammonia-oxidizer abundance

Two months of incubation consecutive to the last NPs application in soil columns, potential nitrification and AOA and AOB abundances were measured. When considering the whole column (i.e. the 4 soil layers), the acute exposure (i.e. 1 exposure at 50 mg L^{-1}) resulted in a significant increase of the AOA abundance (Figure 1; Table 4) but no NPs effect was observed on AOB abundance and potential nitrification activity (Figure 1; Table 4). After 2 NPs exposures at 25 mg L^{-1}, neither the potential nitrification nor the ammonia-oxidizer abundances were affected by TiO_2-NPs contamination (Figure 1, Table 4). In contrast, potential nitrification, AOA and AOB abundances were significantly decreased after 3 applications of TiO_2-NPs at 16.7 mg L^{-1} (Figure 1; Table 4).

Table 4 *P*-values from an ANOVA analysis of the effects of TiO_2-NPs, soil depth and their interaction on potential nitrification, AOA and AOB abundances for each exposure scenario. Values in bold are significant at $P < 0.05$.

		Potential nitrification	AOA	AOB
1 exposure	**TiO_2**	0.33	**<0.001**	0.66
	Depth	0.59	0.64	0.19
	TiO_2 x Depth	0.71	0.74	0.93
2 exposures	**TiO_2**	0.38	0.74	0.34
	Depth	**0.02**	0.59	0.60
	TiO_2 x Depth	0.38	0.60	0.42
3 exposures	**TiO_2**	**0.005**	**0.05**	**0.01**
	Depth	**0.003**	0.99	0.91
	TiO_2 x Depth	0.35	0.97	0.87

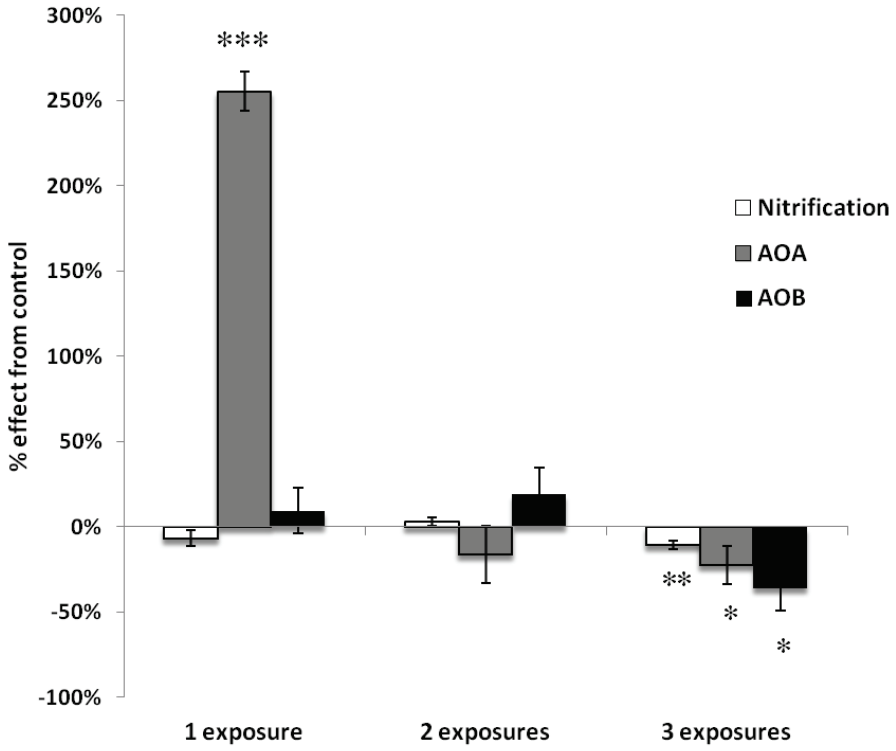


Figure 1 Effect of TiO_2-NPs on potential nitrification activity, AOA and AOB abundance in the whole column (average of the 4 depths). Data are expressed as the percentage change from the control treatment for each exposure. Standard errors are presented (n=6). Significant effects are indicated (***, *P*<0.001; **, *P*<0.01; *, *P*<0.05).

A significant effect of soil depth was only observed on potential nitrification after 2 and 3 exposures (P=0.02 and P=0.003, respectively; Table 4) and no significant interaction between TiO_2-NPs contamination and soil depth was found (Table 4). After 2 exposures, potential nitrification differed significantly according to soil depth, especially at 2 - 4 cm, but no significant effect of the interaction between TiO_2-NPs and soil depth was observed (Figure 2). After 3 exposures, potential nitrification was significantly more reduced by TiO_2-NPs in the first 2 cm than in 2 – 4 and 4 – 6 cm soil depths (Figure 2).

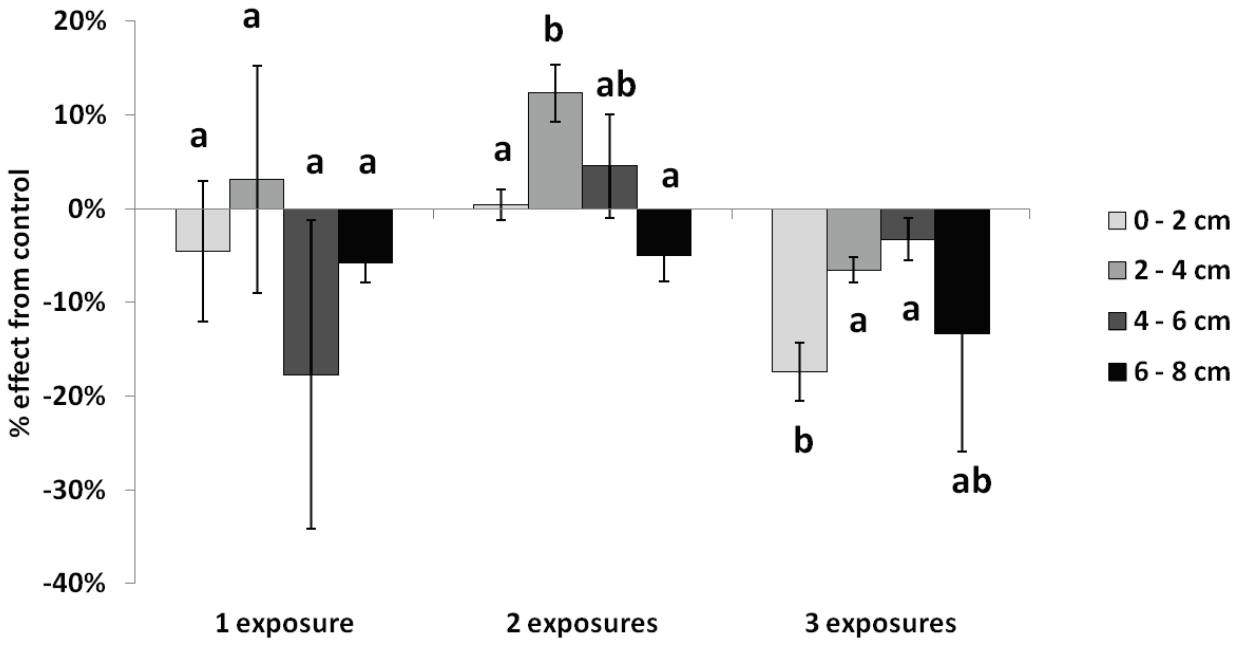


Figure 2 Effect of TiO_2-NPs on potential nitrification activity measured in the four soil layers (depths 0-2 cm; 2-4 cm; 4-6 cm; 6-8 cm) after 1, 2 or 3 soil exposures. Data are expressed as percentage of change from the control treatment for each exposure condition. Standard errors are presented (n=6). For each exposure scenario, bars labeled with the same letter do not differ at $P < 0.05$.

DISCUSSION

Consequences of acute and chronic exposures on TiO_2-NPs transport in soil

Our experimental design enabled the study of the mobility of TiO_2-NPs under acute and chronic exposures for different input concentrations. The results showed that the transport of TiO_2-NPs in soil is influenced by the concentration injected in soil columns for the acute

contaminations. TiO_2 retention increased when higher concentrations were applied. Similar behavior of silica NPs have been observed in sand column by Vitorge *et al.,* (2013) who showed that NPs mobility increased at low concentration regimes. These results can be explained by the increase of TiO_2-NPs aggregation with concentration, which can favor the deposition and straining of these colloids (Solovitch *et al.*, 2010; Cornelis *et al.*, 2014).

Previous studies on TiO_2-NPs mobility in soil reported contrasting results. No transport was observed using 1 g L^{-1} input concentration of TiO_2-NPs (Nickel *et al.*, 2015), while a significant transport was recorded in soils with low clay content and ionic strength using different concentration of TiO_2-NPs in the different soils studied (Fang *et al.*, 2009). Here we showed that despite the fine texture of the soil (39 % clay and 51 % loam), the transport of TiO_2-NPs can occur after an acute exposure, especially under a low concentration regime which is more likely to be observed in natural soils. In the case of an accidental spill for which a high concentration of NPs is expected, the mobility of TiO_2-NPs will be likely very low, in agreement with a classical blocking mechanism typically observed at high NPs concentrations (Vitorge *et al.*, 2013).

For chronic exposures in the soil columns, a very low mobility of TiO_2-NPs was observed and no concentration effect was identified contrarily to the acute exposure scenario. Although TiO_2-NPs distribution along the soil profiles could not be measured (Nickel *et al.*, 2015), it is likely that most of the TiO_2-NPs were retained in first centimeters of the soil (Navarro *et al.*, 2011). This preferential accumulation at the column inlet could have led to pore occlusion and to stronger filtration of the second and third applications of NPs during chronic exposure. These results observed in small columns of 10 cm length suggest that in soils chronically exposed to TiO_2-NPs, these contaminants will accumulate in the first centimeters of the soil and transport to deeper soil layers and groundwater will probably be limited.

Deleterious effects on nitrification after chronic exposure

Two months after the last TiO_2-NPs application, significant decreases of potential nitrification and ammonia-oxidizer abundance were observed only in soil samples exposed 3 times to NPs. These soil columns were exposed to the lowest TiO_2-NPs concentration (16.7 mg L^{-1}) and the final TiO_2-NPs concentration in soil was slightly lower than in the two other

exposure scenarios. Altogether these results showed for the first time that a soil chronic exposure to NPs could be more toxic for soil microbial community and soil functioning than acute exposures. Furthermore, deleterious effects on nitrification being observed only in soil exposed to 3 applications of low concentrations of NPs suggests that resistance and/or resilience of the microbial community to TiO_2-NPs decreases under short term chronic exposure. Indeed in this experiment, the NPs were applied at intervals of 15 days and the effects on nitrification were assessed after a 2 months incubation period. It is well known that a microbial community can develop a tolerance to various stressors over time through modifications of community composition resulting in the selection of tolerant populations and the dispersal of specific resistance via mobile genetic elements (Bissett *et al.*, 2013; Griffiths and Philippot, 2013; Allison and Martiny, 2008). It is likely that chronic exposures occurring on the long term will have less consequences on nitrification because of potential microbial community adaptation over time, as it was observed by Mertens *et al.*(2009) after 2 years of exposure to zinc pollution. To get a better knowledge of NPs impact on soil functioning after chronic contamination, it would be interesting to study the NPs effects on microbial activity, abundance and diversity after each exposure and before each new exposure, in order to monitor the microbial community resistance and to assess resilience, respectively. This approach may also lead to new insights on sensitive and tolerant microbial species among nitrifiers and thus on TiO_2-NPs toxicity mechanisms in soil.

After 3 NPs applications, it is likely that most of TiO_2-NPs were retained on the top of the column. Although a TiO_2 concentration gradient may be present along the soil column profile, no effect of depth was noticed on ammonia-oxidizer abundance that were decreased similarly at the 4 soil depths. Nitrification was significantly more decreased in the first 2 cm and the last 2 cm than in the 2 – 4 and 4 – 6 cm soil layers. With common pollutants it is usually expected that high concentrations have the most deleterious effects. In the case of NPs it has been demonstrated that TiO_2-NPs concentrations induce nonlinear dose-responses on potential nitrification, as well as on AOA and AOB abundance (Article 5). Very low (0.05 mg kg^{-1}) and high concentrations (100 – 500 mg kg^{-1}) can result in similar decreases, which could likely explain the absence of clear effect of soil depth on the response of nitrifiers to TiO_2 applications. These results suggest that despite the low TiO_2 mobility in soil, microbial community can be affected by TiO_2-NPs at the depths of

preferential NPs accumulation (top soil) but also at depths reached by NPs at lower concentration.

CONCLUSIONS

The multidisciplinary approach developed in this study enabled to compare the impact of realistic acute and chronic TiO_2-NPs contaminations in soil columns. The transport of TiO_2-NPs in the studied silty-clay soil was very low for both acute and chronic exposures and varied with NPs injected concentration. In these conditions, the transport of TiO_2-NPs to deeper soil layers or groundwater is likely limited.

Although the chronic exposure resulted in a lower final TiO_2-NPs concentration, it was more toxic to soil microbial community and soil functioning compared to the acute exposure. Chronic exposure in short and long term experiment representing realistic scenarios of soil exposure to NPs are needed to study the resistance and resilience of soil microbial communities to these emerging contaminants and their subsequent consequences on soil fertility.

ACKNOWLEDGMENTS

Marie Simonin was supported by a Ph.D grant from Rhône-Alpes Region – ARC Environnement. This work was funded by a grant from the French National Program Microbien EC2CO-CNRS. The authors thank the technical assistance of Sungeun Lee. Potential nitrification measurements were performed at the AME platform (Microbial Ecology UMR5557-USC1364, Lyon) and quantitative PCR at the DTAMB platform (IFR 41, University Lyon 1).

REFERENCES

Allison SD, Martiny JBH. (2008). Resistance, resilience, and redundancy in microbial communities. *PNAS* **105**:11512–11519.

Bissett A, Brown MV, Siciliano SD, Thrall PH. (2013). Microbial community responses to anthropogenically induced environmental change: towards a systems approach. *Ecol Lett* **16**:128–139.

Brar SK, Verma M, Tyagi RD, Surampalli RY. (2010). Engineered nanoparticles in wastewater and wastewater sludge – Evidence and impacts. *Waste Management* **30**:504–520.

Cornelis G, Hund-Rinke K, Kuhlbusch T, Brink N van den, Nickel C. (2014). Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. *Critical Reviews in Environmental Science and Technology* **44**:2720–2764.

Dassonville N, Guillaumaud N, Piola F, Meerts P, Poly F. (2011). Niche construction by the invasive Asian knotweeds (species complex Fallopia): impact on activity, abundance and community structure of denitrifiers and nitrifiers. *Biol Invasions* **13**:1115–1133.

Dinesh R, Anandaraj M, Srinivasan V, Hamza S. (2012). Engineered nanoparticles in the soil and their potential implications to microbial activity. *Geoderma* **173–174**:19–27.

Fang J, Shan X, Wen B, Lin J, Owens G. (2009). Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. *Environmental Pollution* **157**:1101–1109.

Griffiths BS, Philippot L. (2013). Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiology Reviews* **37**:112–129.

Hooper HL, Jurkschat K, Morgan AJ, Bailey J, Lawlor AJ, Spurgeon DJ, *et al.* (2011). Comparative chronic toxicity of nanoparticulate and ionic zinc to the earthworm Eisenia veneta in a soil matrix. *Environment International* **37**:1111–1117.

Keller AA, McFerran S, Lazareva A, Suh S. (2013). Global life cycle releases of engineered nanomaterials. *J Nanopart Res* **15**:1–17.

Liu R, Lal R. (2015). Potentials of engineered nanoparticles as fertilizers for increasing agronomic productions. *Science of The Total Environment* **514**:131–139.

Mertens J, Broos K, Wakelin SA, Kowalchuk GA, Springael D, Smolders E. (2009). Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. *ISME J* **3**:916–923.

Navarro DA, Banerjee S, Watson DF, Aga DS. (2011). Differences in Soil Mobility and Degradability between Water-Dispersible CdSe and CdSe/ZnS Quantum Dots. *Environ Sci Technol* **45**:6343–6349.

Nickel C, Gabsch S, Hellack B, Nogowski A, Babick F, Stintz M, *et al.* (2015). Mobility of coated and uncoated TiO2 nanomaterials in soil columns–Applicability of the tests methods of OECD TG 312 and 106 for nanomaterials. *Journal of environmental management* **157**:230–237.

Pan B, Xing B. (2012). Applications and implications of manufactured nanoparticles in soils: a review. *European Journal of Soil Science* **63**:437–456.

R Core Team. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing. http://www.R-project.org/.

Rotthauwe JH, Witzel KP, Liesack W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* **63**:4704–4712.

Servin A, Elmer W, Mukherjee A, Torre-Roche RD la, Hamdi H, White JC, *et al.* (2015). A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. *J Nanopart Res* **17**:1–21.

Simonin M, Guyonnet JP, Martins JMF, Ginot M, Richaume A. (2015). Influence of soil properties on the toxicity of TiO2 nanoparticles on carbon mineralization and bacterial abundance. *Journal of Hazardous Materials* **283**:529–535.

Simonin M, Richaume A. (2015). Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review. *Environ Sci Pollut Res* 1–14.

Solovitch N, Labille J, Rose J, Chaurand P, Borschneck D, Wiesner MR, *et al.* (2010). Concurrent Aggregation and Deposition of TiO2 Nanoparticles in a Sandy Porous Media. *Environ Sci Technol* **44**:4897–4902.

Sun TY, Gottschalk F, Hungerbühler K, Nowack B. (2014). Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environmental Pollution* **185**:69–76.

Tourna M, Freitag TE, Nicol GW, Prosser JI. (2008). Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environmental Microbiology* **10**:1357–1364.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk H-P, Schleper C. (2005). Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environmental Microbiology* **7**:1985–1995.

Vitorge E, Szenknect S, Martins JMF, Gaudet J-P. (2013). Size- and concentration-dependent deposition of fluorescent silica colloids in saturated sand columns: transport experiments and modeling. *Environ Sci: Processes Impacts* **15**:1590–1600.

5. Conclusions du chapitre

Dans ce chapitre, nous avons montré que la nitrification était un processus microbien sensible à une pollution aux TiO_2-NPs, même pour des concentrations très faibles (0,05 mg kg^{-1}) et dans des contextes d'exposition aigüe ou chronique. Les diminutions de l'activité nitrifiante ont été expliquées par une forte sensibilité des AOA à ce contaminant et à leur rôle clé dans l'oxydation de l'ammonium dans le sol étudié. Dans ce sol, les AOA étaient principalement représentées par un OTU affilié au genre *Nitrososphaera* mettant en évidence une faible diversité et redondance fonctionnelle au sein de ce groupe. Toutefois, il existe une redondance fonctionnelle entre les AOA et les AOB, ainsi qu'au sein des NOB, qui semble limiter les effets délétères du TiO_2 sur l'activité nitrifiante globale.
Les effets négatifs observés sur l'activité dénitrifiante sont des effets indirects liés à la diminution de l'activité nitrifiante en amont. Le constat réalisé pour d'autres polluants indiquant une plus grande résistance des microorganismes dénitrifiants (comparés aux nitrifiants) semble être vérifiée également dans le cas des TiO_2-NPs.

Nos travaux sur l'écotoxicologie des TiO_2-NPs, se basant sur la nitrification comme indicateur, ont permis de montrer qu'il n'existait pas de relation dose-réponse linéaire contrairement à ce qui peut être observé dans le cas de polluants solubles, tels que les métaux lourds ou les pesticides (Giller et al. 1998 ; Guo et al. 2011). Ce fait est expliqué par l'influence de la concentration utilisée sur les caractéristiques physico-chimiques des NPs, en particulier au niveau de leur agrégation et de leur potentiel oxydant. Ces paramètres sont probablement impliqués directement dans la biodisponibilité et toxicité des TiO_2-NPs qui reposent notamment sur leur capacité à générer des ROS et à s'adsorber à la surface des membranes microbiennes. Le fait que les propriétés des NPs varient avec la concentration utilisée incite à réaliser une caractérisation de ces polluants à toutes les doses testées, contrairement à ce qui est fait habituellement. De plus, il ne semble pas pertinent, dans le cas des NPs, de baser l'évaluation des risques dans les sols sur des relations dose-réponse à partir desquelles sont calculées des EC50, LC50 ou d'autres concentrations définissant des seuils de toxicité.

Nous avons pu voir également que les phénomènes liés à la concentration des NPs ont des conséquences sur leur transport dans les sols, en conséquence d'une modification de

l'agrégation et de leur réactivité vis-à-vis des surfaces réactives du sol. Les NPs les moins agrégées étant plus mobile dans la porosité du sol.

Enfin, nous avons montré qu'une exposition chronique des sols aux NPs était plus toxique pour les microorganismes nitrifiants qu'une exposition aigüe lors d'une contamination en colonne. Ces premiers résultats suggèrent que la résistance et/ou la résilience des communautés microbiennes sont affectées par le nombre d'expositions réalisées et il serait intéressant d'étudier les modifications de structure de communauté, d'abondance et d'activité au cours de chacune de ces expositions.

Nous avons pu aussi constater que le mode d'exposition utilisé pour contaminer le sol avec les TiO_2-NPs a son importance. En effet, une contamination aigüe à 364 mg kg^{-1} effectuée à la surface du sol en colonne n'a pas eu d'effet négatif sur la nitrification (Article 6), alors qu'en microcosmes du même sol lors d'une contamination aigüe « homogène » à 100 et 500 mg kg^{-1}, l'activité était réduite d'environ 25% (Article 5). Ainsi lors de contaminations aigües dans des conditions plus réalistes d'exposition, nos résultats indiquent que les TiO_2-NPs à forte concentration n'auraient plus d'effets délétères sur le fonctionnement microbien du sol étudié.

Chapitre 4 : Conclusions générales et perspectives

L'objectif de ces travaux était d'évaluer les risques associés à la dissémination de NPs d'oxydes métalliques dans les sols, au travers d'une approche pluridisciplinaire se basant sur l'écotoxicologie, l'écologie fonctionnelle et l'écodynamique de ces contaminants. Les indicateurs écologiques choisis ont permis de rendre compte des effets à l'échelle de communautés naturelles d'organismes réalisant des processus clés impliqués dans des services écosystémiques rendus par les sols. Différentes questions ont été adressées au début de la thèse et dans cette partie conclusive, nous reviendrons sur chacune d'entre elle afin de tirer des conclusions générales et de proposer des perspectives de recherche.

1. Influence des propriétés du sol sur le devenir et l'écotoxicité des NPs d'oxydes métalliques

- **Quelle est l'influence des propriétés du sol, notamment la texture et la teneur en matière organique, sur la mobilité des NPs d'oxydes métalliques ?**

Globalement, la mobilité du TiO_2 et CuO observée dans les 6 sols est très faible car au minimum 85% des NPs injectées sont retenues dans le sol. Comme attendu, nous avons constaté que les propriétés du sol influençaient les caractéristiques physico-chimiques et par conséquent le transport des NPs d'oxydes métalliques. Cependant, contrairement à ce qui peut être observé avec des polluants solubles, nous n'avons pas pu dégager une typologie de sol dans laquelle la mobilité des NPs serait favorisée, en raison de l'absence de relation claire entre la texture du sol et la mobilité des NPs. En se basant sur la littérature, on pouvait s'attendre à un transport plus important dans les sols sableux, mais que l'on considère les NPs de TiO_2 ou de CuO, cela n'a pas été vérifié, ce qui en soit est un résultat très intéressant. La teneur en argile du sol ne semble donc pas être un paramètre clé du transport des NPs dans les sols étudiés. En revanche, la teneur en MO apparait comme un paramètre d'intérêt, en particulier le carbone organique dissous qui influence l'agrégation, les charges de surface et au final la dispersion et la mobilité des CuO-NPs dans les sols. Le transport du CuO était accru dans des sols ayant des concentrations en carbone organique dissout élevées indépendamment de la texture (sablo-limoneux et limono-argileux).

Ces résultats montrent la nécessité d'étudier plus spécifiquement le rôle de la MO dissoute, en termes de qualité et de quantité, sur les caractéristiques des NPs et leur mobilité dans les

sols. Des analyses sont en cours pour caractériser la MO des sols et solutions de sol utilisées, par spectroscopie en proche infrarouge (collaboration Lauric Cecillon-IRSTEA Grenoble).

L'étude du TiO_2 dans les sols est très complexe du fait de la forte teneur en titane des sols et des solutions de sols. De ce fait, les courbes de percée n'étaient pas exploitables pour la comparaison entre sols et pour la modélisation. Toutefois, nous avons pu mettre en évidence que la concentration en TiO_2-NPs utilisée a des conséquences pour leur transport dans les sols, certainement via une modification de leur agrégation, les TiO_2-NPs les moins agrégées étant plus mobiles dans la porosité du sol.

Pour améliorer les connaissances du transport du TiO_2 dans les sols, l'utilisation de nanotraceurs contenant un marqueur permettant leur détection (e.g. fluorescents, radioactifs, magnétiques ; Vitorge, 2010 ; Hildebrand et Franke, 2012) serait une solution à considérer.

L'étude de l'influence de la concentration en NPs sur leur transport dans différents sols naturels sera également à poursuivre dans de futures études. Cet aspect est d'autant plus important qu'en fonction du mode d'exposition des sols, par l'intermédiaire d'épandage de boues, l'utilisation de NPs dans des produits sanitaires ou fertilisants ou encore lors d'un déversement accidentel, la concentration en NPs sera très différente, induisant sans doute des comportements différents.

A l'heure actuelle, il reste de nombreuses zones d'ombres concernant le devenir des NPs dans les sols. Toutefois, il est clair que les NPs sont très peu mobiles dans les sols et sont donc susceptibles de persister pendant de longues durées dans cet écosystème. Ce contexte implique la prise en considération de leur vieillissement/transformation au cours du temps, mais aussi de leur remobilisation dans les sols sur des échelles de temps beaucoup plus longues que celles généralement étudiées, ce qui ouvre donc également des perspectives intéressantes.

- **Quelle est l'influence des propriétés du sol, en particulier de la texture et de la teneur en matière organique, sur la toxicité des NPs d'oxydes métalliques sur les communautés microbiennes du sol?**

Les TiO_2-NPs n'ont pas induit d'effets toxiques sur les communautés microbiennes dans la majorité des sols. Des effets délétères sur le fonctionnement microbien n'ont été observés que dans un sol sur les six étudiés. Il s'agit du sol limono-argileux (forte MO) qui présentait le pH et la teneur en MO les plus élevés. Ces résultats peuvent paraître surprenants car il a été mis en évidence dans la littérature que les communautés microbiennes de sols ayant des teneurs élevées en argile et MO étaient plus résistants et résilients aux perturbations, en particulier aux métaux lourds (Giller *et al.,* 1998 ; Debeljak *et al.,* 2009 Griffiths et Philippot 2013). Dans cette étude, le résultat inverse a donc été observé, mettant en évidence que les connaissances issues de l'étude des métaux lourds ne sont pas transposables simplement aux NPs métalliques.

Dans la solution du sol limono-argileux, les TiO_2-NPs avaient des caractéristiques physico-chimiques singulières comparées aux autres sols, pouvant probablement expliquer leur plus forte toxicité. En effet, en raison des caractéristiques de la solution du sol (force ionique, pH, carbone organique dissous, calcium), les TiO_2-NPs étaient moins stables en suspension. Il est vraisemblable que dans ces conditions, l'hétéroagrégation soit favorisée ce qui peut conduire à l'adsorption des NPs sur les membranes microbiennes. Ces résultats montrent que les interactions complexes entre NPs et composés du sol rendent difficiles la mise en évidence des facteurs favorisant leur toxicité. Des études complémentaires seraient nécessaires pour déterminer l'influence de la stabilité des TiO_2-NPs sur leur biodisponibilité et donc leur toxicité dans les sols. Une démarche réductionniste consistant à évaluer la toxicité des NPs dans des conditions simplifiées en solution de sol serait sans doute adaptée pour tester cette hypothèse.

Il serait également intéressant de tester les effets des TiO_2-NPs sur un plus grand nombre de sols afin d'avoir une puissance statistique importante pour évaluer l'influence des propriétés du sol sur leur toxicité. Dans le panel de sols choisi, il faudrait sélectionner des sols qui présentent des caractéristiques très contrastées, mais également des sols aux propriétés similaires, afin de pouvoir différencier les effets liés aux paramètres physico-chimiques et ceux liés à la sensibilité de certaines communautés microbiennes vis-à-vis de ce polluant.

Cela permettrait en particulier de déterminer si le pH et la teneur en MO sont réellement des paramètres dominants influençant la biodisponibilité et toxicité du TiO_2.

Les effets observés sur les indicateurs microbiens reposent sur l'influence des propriétés du sol sur la toxicité du polluant mais également sur la résistance de la communauté microbienne exposée. Les effets délétères du TiO_2 dans le sol limono-argileux pourraient donc être une conséquence de la plus grande sensibilité de la communauté microbienne de ce sol comparée à celle des autres sols. Pour vérifier cela, on pourrait envisager d'extraire des communautés microbiennes à partir de différents sols puis de les exposer aux TiO_2-NPs dans un même environnement afin de comparer leur résistance.

En conclusion, les propriétés du sol influencent le devenir et la toxicité des NPs, mais à ce stade des recherches, il ne parait pas possible d'identifier une typologie de sols présentant une vulnérabilité particulière aux NPs. Au vu de la complexité des interactions et des transformations subies par les NPs dans les sols et des nombreux facteurs qui conditionnent la réponse des communautés microbiennes, il serait même légitime de s'interroger sur le réalisme de cet objectif. Bien que les effets toxiques des TiO_2-NPs aient été mis en évidence dans un seul sol sur les six étudiés, le principe de précaution inciterait à ce qu'une réglementation en faveur de la protection des sols soit proposée pour ce type de composés, notamment car leur comportement et toxicité dans les sols est difficilement prévisible à l'heure actuelle.

2. Impact des TiO_2-NPs sur le cycle de l'N

- **Quel est l'effet de ces NPs sur les groupes fonctionnels microbiens nitrifiants et dénitrifiants impliqués dans le cycle de l'azote dont les niveaux de redondance fonctionnelle sont différents?**

Dans le sol limono-argileux, des effets délétères sur les activités microbiennes du cycle de l'N ont été observés. Comme nous en avions fait l'hypothèse, le processus microbien le plus sensible aux TiO_2-NPs est la nitrification du fait de la faible redondance fonctionnelle des groupes fonctionnels microbiens impliqués. En conséquence de la diminution de l'activité nitrifiante, la dénitrification était réduite mais dans une moindre mesure et aucune

modification de l'abondance des dénitrifiants n'a été observée. Nos résultats suggèrent que les TiO_2-NPs peuvent avoir des conséquences globales sur le cycle de l'N, en particulier sur la disponibilité en NH_4^+ et NO_3^- qui sont 2 sources clés pour la nutrition végétale.
Dans le futur, un changement d'échelle d'étude sera nécessaire afin (i) d'évaluer les conséquences indirectes des effets sur le cycle de l'N sur la croissance végétale et (ii) d'étudier comment les plantes modulent les effets des NPs sur les communautés microbiennes, en particulier via l'exsudation racinaire car ces composés organiques pourraient modifier la biodisponibilité et la toxicité des NPs (Martineau *et al.,* 2014). Un projet permettant d'aborder ces questions en utilisant des mésocosmes plantés a été déposé à la fondation Rovaltain (RT2E-2015) afin de poursuivre les investigations initiées dans ce travail.

Dans le sol étudié, les AOA semblent être un groupe microbien « clé de voute » pour le fonctionnement du cycle de l'azote. Paradoxalement, ce groupe fonctionnel se caractérise par une très faible diversité avec la dominance du genre *Nitrososphaera* et donc une très faible redondance fonctionnelle pour la réalisation de l'oxydation de l'ammonium. Suite à ce constat, on peut se demander si les sols dans lesquels la nitrification repose principalement sur l'activité des AOA sont moins résistants et résilients aux perturbations, et aux polluants en particulier. En effet, la faible diversité des archées totales et AOA a été mise en évidence dans différentes études (Bates *et al.,* 2010 ; Pester *et al.,* 2012) et il a été montré que la restauration de l'activité nitrifiante dans un sol contaminé au Zn était assuré par les AOB et pas les AOA (Mertens *et al.,* 2009). Il faut toutefois noter que les outils moléculaires pour étudier les archées, notamment les amorces de PCR, sont en constante amélioration et évaluation et qu'il est possible que les résultats obtenus à l'heure actuelle ne reflète pas la diversité complète de cette communauté (Pester *et al* ., 2012). Par ailleurs, le faible nombre de souches isolées du sol (Lehtovirta-Morley *et al.,* 2011 ; Tourna *et al.,* 2011) limite la connaissance sur la physiologie et l'écologie de ces procaryotes (Prosser et Nicol, 2012). La différenciation de niches entre AOA et AOB n'est pas encore claire et il est vraisemblable qu'elle repose sur une combinaison de facteurs environnementaux, tels que le pH, la MO et la concentration en ammonium (Erguder *et al.,* 2010 ; Prosser et Nicol, 2012 ; Zhalnina *et al.,* 2012). Au vu des différents niveaux de diversité présents dans ces 2 groupes, on pourrait se demander si le degré de perturbation d'un sol (e.g. pollutions, pratiques agricoles…) ne

serait pas un paramètre supplémentaire à prendre en considération pour comprendre l'abondance relative et le rôle fonctionnel des AOA et AOB dans les sols (Vico Oton *et al.,* 2015). Pour étayer cette hypothèse, il conviendrait dans un premier temps d'identifier des sols non-perturbés dans lesquels les AOA ont un rôle fonctionnel majeur, puis d'exposer ces sols à différents types de perturbations pour déterminer si cela conduit à ce que les AOB deviennent dominants.

L'approche statistique par path analysis a contribué à révéler les effets directs et indirects des NPs, ainsi que les liens fonctionnels entre les différents acteurs de la nitrification et dénitrification. Cette approche offre une vision intégrée de l'impact d'un polluant sur les communautés microbiennes et leur fonctionnement. Elle a permis de mettre en évidence les effets négatifs en cascades dus à la sensibilité des AOA du genre *Nitrososphaera*, ainsi que les altérations de la nitrification expliquées par des modifications de couplage entre les groupes fonctionnels impliqués dans la première et la seconde étape du processus. Ces résultats confirment l'intérêt en écotoxicologie d'une approche systémique intégrant la structure des communautés, les activités, les fluctuations temporelles, les interactions et les feedbacks (Bisset *et al.,* 2013).

Nous n'avons pas intégré de facteurs abiotiques (T°C, humidité, pH…) dans les path analysis en raison de leur stabilité dans les microcosmes. Cependant, dans des expérimentations en mésocosmes, il sera intéressant d'intégrer plus de variables abiotiques et biotiques d'intérêt dans ce type d'approche statistique en vue d'élucider la complexité du système. Certains aspects non étudiés dans la thèse pourront être abordés, tels que les effets indirects des NPs sur les propriétés du sol via la modification de la chimie de la solution du sol ou la remobilisation de polluants (Ben Moshe *et al.,* 2013 ; Priester *et al.,* 2013 ; McShane *et al.,* 2014).

Les groupes fonctionnels nitrifiants sont donc des indicateurs microbiens sensibles à la présence des TiO_2-NPs dans les sols qui peuvent entrainer des conséquences plus larges sur la dénitrification. Ces résultats questionnent quant à l'impact global des TiO_2-NPs sur le fonctionnement des écosystèmes, car dans cette étude nous nous sommes intéressés uniquement aux processus de minéralisation du carbone, de nitrification et de dénitrification. Nos résultats suggèrent que le service écosystémique de maintenance de la

fertilité par les communautés microbiennes pourrait être altéré, mais des expérimentations à plus grande échelle doivent encore être menées pour valider ces conclusions.

- **Les faibles concentrations ont-elles des effets négatifs sur le fonctionnement microbiologique du sol ? Existe-t-il une relation dose-réponse associée à l'impact des NPs sur les communautés microbiennes du sol ?**

Des effets négatifs de très faibles concentrations en TiO_2-NPs (0.05 mg kg^{-1}) ont été mis en évidence sur l'activité nitrifiante dans le sol limono-argileux. Cependant, en étudiant l'effet de ce contaminant sur différents indicateurs microbiens, aucune relation dose-réponse « classique » n'a été observée. Ceci semble être expliqué par l'influence de la concentration utilisée sur les caractéristiques physico-chimiques des NPs, en particulier au niveau de leur agrégation et de leur potentiel oxydant. Ainsi, de façon contre-intuitive, une forte concentration en NPs pourrait être moins biodisponible et moins toxique qu'une faible concentration dans les sols. Ce résultat remet donc en question les approches d'évaluation des risques des NPs dans les sols par la définition de concentrations d'effets et de concentrations seuils (EC_{50}, LOEC…) qui se basent sur les relations dose-réponses. Pour le moment, les expérimentations ayant été effectuées dans un seul sol, il est nécessaire de renouveler l'étude des relations dose-réponse dans des sols présentant des propriétés contrastées pour confirmer ce résultat. Dans ce contexte, l'étude de la toxicité de gammes très larges de concentrations sera à vérifier en intégrant des concentrations faibles (1 µg à 1 mg kg^{-1}) qui sont encore peu testées, alors que ces doses de NPs sont prédites dans les sols (Sun *et al.,* 2014).

Sur différents indicateurs, nous avons observé des effets négatifs qui restent stables ou qui augmentent au cours du temps. Les expériences menées durant 90 jours d'exposition ont rarement mis en évidence une résilience des effets. La majorité de la littérature existante a évalué les effets à court terme des NPs (Simonin et Richaume, 2015), de ce fait nous nous attendions plutôt à des effets sur le court terme, puis à une résilience suite au vieillissement des NPs dans les sols et la sélection de populations tolérantes. Les taux de croissance des micro-organismes dans les sols sont faibles (Demoling *et al.,* 2007), comparés à ceux mesurés *in vitro*, particulièrement dans le cas des micro-organismes autotrophes impliqués dans la nitrification. Ce fait pourrait expliquer pourquoi certains effets sur l'abondance ou la

diversité ne sont détectés qu'après 90 jours. De plus, l'ADN extrait peut provenir de cellules mortes en raison de sa persistance possible pendant plusieurs semaines dans le sol (Niemeyer et Gessler, 2002), ce qui a pu empêcher la détection d'effets à court terme sur l'abondance et la diversité microbienne. Des études sont encore nécessaires pour évaluer les effets des NPs dans les sols sur le long terme (plusieurs mois ou années) suite à leur transformation/vieillissement et établir si une résilience des effets est possible et dans quel délai.

3. Effet d'une pollution chronique

- **Le transport et la toxicité des NPs dans les sols sont-ils modifiés lors d'apports chroniques et aigus ?**

Les contaminations chroniques conduites en colonnes de sols ont montré que les NPs sont mobiles majoritairement lors de la première contamination et qu'elles sont par la suite retenues en quasi-totalité dans le sol. De ce fait pour une même concentration injectée, nous avons mis en évidence que la concentration en TiO_2-NPs retenue dans le sol est plus grande dans le cas de contamination aigüe que lors d'une contamination chronique. Toutefois, les effets délétères sur la nitrification ont été observés uniquement dans le cas de trois expositions successives aux NPs résultant en une concentration en TiO_2-NPs plus faible dans le sol que dans les autres scénarios d'exposition. Ces observations fournissent des éléments nouveaux qui méritent d'être pris en compte en matière de risques associés au NPs dans les sols. Il s'agit en effet d'une toute première étude mettant en évidence que les expositions chroniques sont plus toxiques que les expositions aigües pour les communautés microbiennes. Dans un contexte où les épandages de boues, l'irrigation ou l'utilisation de nanofertilisants engendrent vraisemblablement une exposition chronique des sols agricoles, un tel constat mérite une attention particulière.

Il est donc nécessaire de poursuivre ce travail en considérant d'autres sols, une gamme de concentrations testées plus large, des durées d'expositions plus longues, d'autres indicateurs et un nombre de contaminations plus important afin de valider la généricité de ces résultats. Un transfert d'échelle expérimentale, permettant de considérer une distance de parcours plus réaliste que les 10 cm utilisés, sera également une étape indispensable.

Le mode de contamination des sols est également un élément clé à considérer dans le futur. En effet, une contamination du sol « homogène » dans les microcosmes et une contamination apportée en surface dans une colonne ont des conséquences différentes sur la nitrification. La contamination aigüe en colonne de sol n'a eu aucun effet sur la nitrification contrairement à ce qui a été observé en microcosmes, alors que le sol utilisé pour ces expériences avait été prélevé au même moment. De ce fait, il est possible que les expositions réalisées en microcosmes ne soient pas suffisamment représentatives de la toxicité des NPs dans cet environnement. La très forte réactivité des NPs nécessite d'adapter les protocoles d'analyse de l'écotoxicité des NPs dans les sols. Nos résultats vont dans ce sens, ce qui incite donc à mettre en œuvre des systèmes expérimentaux s'approchant au mieux de conditions réalistes d'expositions afin d'améliorer la fiabilité de l'évaluation des risques associés aux NPs dans le futur.

L'évaluation des risques associés aux NPs nécessiterait à moyen terme la réalisation d'études de terrain sur des sites de pollution aux NPs (usine, déversement accidentel…), afin d'étudier directement l'effet d'une contamination aigüe ou chronique sur le long terme dans des conditions naturelles. Ces suivis de terrain permettraient de valider la pertinence des différents indicateurs microbiens utilisés dans la thèse et plus généralement dans la littérature. Ils se révéleraient également très utiles pour caractériser les NPs et leur transport *in situ*. De plus, ces sols contaminés pourraient être collectés pour être à nouveau exposés au laboratoire afin d'étudier la résilience et la résistance des communautés microbiennes, et identifier des communautés adaptées à ces perturbations.

La problématique des NPs se pose également dans un cadre plus large de multipollutions qui constituent un des défis scientifiques majeurs actuels. Il conviendrait donc d'envisager leur impact en association avec d'autres polluants comme les métaux lourds, les pesticides ou les antibiotiques, dans la mesure où ces derniers partagent les mêmes voies d'entrées dans le sol, par exemple via les boues de station d'épuration. Les interactions entre les NPs et d'autres polluants font déjà l'objet d'applications, par exemple en remédiation ou retraitement des eaux (Fang *et al.,* 2011 ; Hua *et al.,* 2012). Il est donc probable que la toxicité de tels cocktails ne résulte pas seulement d'effets additifs, mais plus vraisemblablement d'effets synergiques ou antagonistes associés à des interactions complexes.

4. Une approche « systems biology » au service de l'écotoxicologie

Durant les 20 dernières années, les approches de type « systems biology » ont émergé comme une discipline scientifique holistique qui vise à comprendre comment les interactions s'opérant à différents niveaux d'organisation biologique (e.g. de l'ADN/ARN/protéine aux cellules, organismes, jusqu'à la structure des communautés et le fonctionnement) et les paramètres environnementaux (e.g. nutriments, humidité, jusqu'aux conditions climatiques locales et globales) donnent naissance aux processus biologiques et écologiques (Bisset *et al.,* 2013 ; Raes et Bork, 2008). Ces travaux de thèse s'inscrivent en partie dans cette démarche mais en l'appliquant à l'écotoxicologie. Cette approche apporte un éclairage nouveau sur l'écotoxicologie en permettant de rendre compte des effets de perturbations à l'échelle du fonctionnement des communautés, dans le but final de déterminer les impacts sur des services écosystémiques tels que la maintenance de la fertilité du sol et de la biodiversité (Millenium ecosystem assessment, 2005). Ainsi, l'écotoxicologie ne se cantonne plus seulement à rendre compte d'effets sur des variables isolées et à définir des concentrations seuils et des relations dose-réponse, mais tend vers une meilleure compréhension de l'influence de perturbations sur le fonctionnement des écosystèmes en intégrant différentes échelles d'études (cellule, population, communauté) et les interactions biotiques et abiotiques.

L'intégration d'une démarche de type « systems biology » permet ainsi d'orienter l'écotoxicologie vers de l'écologie du stress ce qui répond aux nombreux appels à inclure plus d' « éco » en écotoxicologie (Calow, 1996 ; Van Straalen, 2003) alors que dans les faits, encore actuellement, l'écotoxicologie est plus souvent synonyme de toxicologie environnementale (Van Straalen, 2003).

Dans cette thèse, la démarche intégrative que nous avons adoptée a permis d'apporter une contribution pluridisciplinaire originale pour la compréhension de l'impact des NPs d'oxydes métalliques. Toutefois, la route est encore longue avant que le devenir et la toxicité des nanomatériaux dans les sols soient bien compris et qu'une réglementation adaptée à cet environnement soit proposée.

Références

Auffan M, Rose J, Bottero J-Y, Lowry GV, Jolivet J-P, Wiesner MR. (2009). Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nat Nanotechnol* **4**:634–641.

Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N. (2011). Examining the global distribution of dominant archaeal populations in soil. *ISME J* **5**:908–917.

Ben-Moshe T, Dror I, Berkowitz B. (2010). Transport of metal oxide nanoparticles in saturated porous media. *Chemosphere* **81**:387–393.

Ben-Moshe T, Frenk S, Dror I, Minz D, Berkowitz B. (2013). Effects of metal oxide nanoparticles on soil properties. *Chemosphere* **90**:640–646.

Bondarenko O, Ivask A, Käkinen A, Kahru A. (2012). Sub-toxic effects of CuO nanoparticles on bacteria: Kinetics, role of Cu ions and possible mechanisms of action. *Environ Pollut* **169**:81–89.

Calow P. (1996). Ecology in ecotoxicology: some possible 'rules of thumb'. In:*ECOtoxicology: Ecological Dimensions*, Baird, DJ, Maltby, L, Greig-Smith, PW, & Douben, PET (eds) Chapman & Hall Ecotoxicology Series, Springer Netherlands, pp. 5–12.

Buffle J, Wilkinson KJ, Stoll S, Filella M, Zhang J. (1998). A Generalized Description of Aquatic Colloidal Interactions: The Three-colloidal Component Approach. *Environ Sci Technol* **32**:2887–2899.

Chen X, Mao SS. (2007). Titanium Dioxide Nanomaterials: Synthesis, Properties, Modifications, and Applications. *Chem Rev* **107**:2891–2959.

Chowdhury I, Cwiertny DM, Walker SL. (2012). Combined Factors Influencing the Aggregation and Deposition of nano-TiO2 in the Presence of Humic Acid and Bacteria. *Environ Sci Technol* **46**:6968–6976.

Cornelis G, Hund-Rinke K, Kuhlbusch T, Brink N van den, Nickel C. (2014). Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. *Crit Rev Environ Sci Technol* **44**:2720–2764.

Cornelis G, Pang L, Doolette C, Kirby JK, McLaughlin MJ. (2013). Transport of silver nanoparticles in saturated columns of natural soils. *Sci Total Environ* **463–464**:120–130.

Cornelis G, Ryan B, McLaughlin MJ, Kirby JK, Beak D, Chittleborough D. (2011). Solubility and Batch Retention of CeO2 Nanoparticles in Soils. *Environ Sci Technol* **45**:2777–2782.

Coutris C, Joner EJ, Oughton DH. (2012). Aging and soil organic matter content affect the fate of silver nanoparticles in soil. *Sci Total Environ* **420**:327–333.

Darlington T, Neigh A, Spencer M, Nguyen O, Oldenburg S. (2009). Nanoparticle characteristics affecting environmental fate and transport through soil. *Environmental Toxicology and Chemistry* **28**:1191–1199.

Demoling F, Figueroa D, Bååth E. (2007). Comparison of factors limiting bacterial growth in different soils. *Soil Biology and Biochemistry* **39**:2485–2495.

Diez-Ortiz M, Lahive E, George S, Ter Schure A, Van Gestel CAM, Jurkschat K, *et al.* (2015). Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; bioavailability and toxicity of silver ions (AgNO3) and silver nanoparticles to earthworm Eisenia fetida in long-term aged soils. *Environ Pollut* **203**:191–198.

Elimelech M, Gregory J, Jia X. (2013). Particle Deposition and Aggregation: Measurement, Modelling and Simulation. Butterworth-Heinemann.

Erguder TH, Boon N, Wittebolle L, Marzorati M, Verstraete W. (2009). Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. *FEMS Microbiology Reviews* **33**:855–869.

Espinasse B, Hotze EM, Wiesner MR. (2007). Transport and Retention of Colloidal Aggregates of C60 in Porous Media: Effects of Organic Macromolecules, Ionic Composition, and Preparation Method. *Environ Sci Technol* **41**:7396–7402.

Fang J, Shan X, Wen B, Lin J, Owens G. (2009). Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. *Environmental Pollution* **157**:1101–1109.

Fang J, Shan X, Wen B, Lin J, Owens G, Zhou S. (2011). Transport of copper as affected by titania nanoparticles in soil columns. *Environmental Pollution* **159**:1248–1256.

Giller KE, Witter E, Mcgrath SP. (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biol Biochem* **30**:1389–1414.

Gottschalk F, Sonderer T, Scholz R, Nowack B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science & Technology* **43**:9216–9222.

Griffiths BS, Philippot L. (2013). Insights into the resistance and resilience of the soil microbial community. *Fems Microbiol Rev* **37**:112–129.

Guo G-X, Deng H, Qiao M, Mu Y-J, Zhu Y-G. (2011). Effect of pyrene on denitrification activity and abundance and composition of denitrifying community in an agricultural soil. *Environmental Pollution* **159**:1886–1895.

Handy RD, Kammer F von der, Lead JR, Hassellöv M, Owen R, Crane M. (2008). The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology* **17**:287–314.

Hildebrand H, Franke K. (2012). A new radiolabeling method for commercial Ag0 nanopowder with 110mAg for sensitive nanoparticle detection in complex media. *J Nanopart Res* **14**:1–7.

Holden PA, Schimel JP, Godwin HA. (2014). Five reasons to use bacteria when assessing manufactured nanomaterial environmental hazards and fates. *Curr Opin Biotechnol* **27**:73–78.

Hotze EM, Bottero J-Y, Wiesner MR. (2010). Theoretical framework for nanoparticle reactivity as a function of aggregation state. *Langmuir* **26**:11170–11175.

Hua M, Zhang S, Pan B, Zhang W, Lv L, Zhang Q. (2012). Heavy metal removal from water/wastewater by nanosized metal oxides: A review. *J Hazard Mater* **211–212**:317–331.

INRS. (2012). Les nanomatériaux: Définitions, risques toxicologiques, caractérisation de l'exposition professionnelle et mesures de prévention. **ED6050**.

Jaisi DP, Elimelech M. (2009). Single-Walled Carbon Nanotubes Exhibit Limited Transport in Soil Columns. *Environ Sci Technol* **43**:9161–9166.

Jaisi DP, Saleh NB, Blake RE, Elimelech M. (2008). Transport of Single-Walled Carbon Nanotubes in Porous Media: Filtration Mechanisms and Reversibility. *Environ Sci Technol* **42**:8317–8323.

Jeong S-W, Kim S-D. (2009). Aggregation and transport of copper oxide nanoparticles in porous media. *J Environ Monit* **11**:1595–1600.

Jiang J, Oberdörster G, Biswas P. (2008). Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *J Nanoparticle Res* **11**:77–89.

Jomini S, Labille J, Bauda P, Pagnout C. (2012). Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO2 nanoparticles and byproducts from a sunscreen TiO2-based nanocomposite. *Toxicol Lett* **215**:54–61.

Ju-Nam Y, Lead JR. (2008). Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications. *Sci Total Environ* **400**:396–414.

Kaegi R, Ulrich A, Sinnet B, Vonbank R, Wichser A, Zuleeg S, *et al.* (2008). Synthetic TiO2 nanoparticle emission from exterior facades into the aquatic environment. *Environ Pollut* **156**:233–239.

Kandeler F, Kampichler C, Horak O. (1996). Influence of heavy metals on the functional diversity of soil microbial communities. *Biol Fertil Soils* **23**:299–306.

Kasel D, Bradford SA, Šimůnek J, Pütz T, Vereecken H, Klumpp E. (2013). Limited transport of functionalized multi-walled carbon nanotubes in two natural soils. *Environ Pollut* **180**:152–158.

Keller AA, McFerran S, Lazareva A, Suh S. (2013). Global life cycle releases of engineered nanomaterials. *J Nanoparticle Res* **15**:1–17.

Khin MM, Nair AS, Babu VJ, Murugan R, Ramakrishna S. (2012). A review on nanomaterials for environmental remediation. *Energy Environ Sci* **5**:8075–8109.

Kiser MA, Westerhoff P, Benn T, Wang Y, Pérez-Rivera J, Hristovski K. (2009). Titanium Nanomaterial Removal and Release from Wastewater Treatment Plants. *Environ Sci Technol* **43**:6757–6763.

Kool PL, Ortiz MD, van Gestel CAM. (2011). Chronic toxicity of ZnO nanoparticles, non-nano ZnO and ZnCl2 to Folsomia candida (Collembola) in relation to bioavailability in soil. *Environ Pollut* **159**:2713–2719.

Kowalchuk G, Stephen J. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual Reviews in Microbiology* **55**:485–529.

Labille J, Feng J, Botta C, Borschneck D, Sammut M, Cabie M, *et al.* (2010). Aging of TiO2 nanocomposites used in sunscreen. Dispersion and fate of the degradation products in aqueous environment. *Environ Pollut* **158**:3482–3489.

Leahy JG, Colwell RR. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol Rev* **54**:305–315.

Lecoanet HF, Bottero J-Y, Wiesner MR. (2004). Laboratory Assessment of the Mobility of Nanomaterials in Porous Media. *Environ Sci Technol* **38**:5164–5169.

Lecoanet HF, Wiesner MR. (2004). Velocity Effects on Fullerene and Oxide Nanoparticle Deposition in Porous Media. *Environ Sci Technol* **38**:4377–4382.

Lehtovirta-Morley LE, Stoecker K, Vilcinskas A, Prosser JI, Nicol GW. (2011). Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *PNAS* **108**:15892–15897.

Li Y, Zhang W, Niu J, Chen Y. (2012). Mechanism of Photogenerated Reactive Oxygen Species and Correlation with the Antibacterial Properties of Engineered Metal-Oxide Nanoparticles. *Acs Nano* **6**:5164–5173.

Liu R, Lal R. (2015). Potentials of engineered nanoparticles as fertilizers for increasing agronomic productions. *Sci Total Environ* **514**:131–139.

Loreau M, Naeem S, Inchausti P. (2002). Biodiversity and Ecosystem Functioning: Synthesis and Perspectives. Oxford University Press.

Lowry GV, Gregory KB, Apte SC, Lead JR. (2012). Transformations of Nanomaterials in the Environment. *Environ Sci Technol* **46**:6893–6899.

Martineau N, McLean JE, Dimkpa CO, Britt DW, Anderson AJ. (2014). Components from wheat roots modify the bioactivity of ZnO and CuO nanoparticles in a soil bacterium. *Environmental Pollution* **187**:65–72.

McShane HVA, Sunahara GI, Whalen JK, Hendershot WH. (2014). Differences in Soil Solution Chemistry between Soils Amended with Nanosized CuO or Cu Reference Materials: Implications for Nanotoxicity Tests. *Environ Sci Technol* **48**:8135–8142.

Mertens J, Broos K, Wakelin SA, Kowalchuk GA, Springael D, Smolders E. (2009). Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. *ISME J* **3**:916–923.

Millenium Ecosystem Assessment, others. (2005). Ecosystems and human well-being. Island Press Washington, DC http://www.who.int/entity/globalchange/ecosystems/ecosys.pdf (Accessed August 4, 2015).

Mitrano DM, Motellier S, Clavaguera S, Nowack B. (2015). Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. *Environ Int* **77**:132–147.

Navarro DA, Banerjee S, Watson DF, Aga DS. (2011). Differences in Soil Mobility and Degradability between Water-Dispersible CdSe and CdSe/ZnS Quantum Dots. *Environ Sci Technol* **45**:6343–6349.

Nel A, Xia T, Mädler L, Li N. (2006). Toxic potential of materials at the nanolevel. *Science* **311**:622–627.

Nickel C, Gabsch S, Hellack B, Nogowski A, Babick F, Stintz M, *et al.* (2015). Mobility of coated and uncoated TiO 2 nanomaterials in soil columns–Applicability of the tests methods of OECD TG 312 and 106 for nanomaterials. *J Environ Manage* **157**:230–237.

Niemeyer J, Gessler F. (2002). Determination of free DNA in soils. *Z Pflanzenernähr Bodenk* **165**:121–124.

Nowack B, Ranville JF, Diamond S, Gallego-Urrea JA, Metcalfe C, Rose J, *et al.* (2012). Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environ Toxicol Chem* **31**:50–59.

Ollivier J, Schacht D, Kindler R, Groeneweg J, Engel M, Wilke B-M, *et al.* (2013). Effects of repeated application of sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions. *Front Microbiol* **4**. doi:10.3389/fmicb.2013.00022.

Oton EV, Quince C, Nicol GW, Prosser JI, Gubry-Rangin C. (2015). Phylogenetic congruence and ecological coherence in terrestrial Thaumarchaeota. *ISME J*. doi:10.1038/ismej.2015.101.

Pan B, Xing B. (2012). Applications and implications of manufactured nanoparticles in soils: a review. *Eur J Soil Sci* **63**:437–456.

Pester M, Rattei T, Flechl S, Gröngröft A, Richter A, Overmann J, *et al.* (2012). amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions. *Environmental Microbiology* **14**:525–539.

Petersen EJ, Zhang L, Mattison NT, O'Carroll DM, Whelton AJ, Uddin N, *et al.* (2011). Potential Release Pathways, Environmental Fate, And Ecological Risks of Carbon Nanotubes. *Environ Sci Technol* **45**:9837–9856.

Piccinno F, Gottschalk F, Seeger S, Nowack B. (2012). Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *J Nanoparticle Res* **14**:1–11.

Priester JH, Ge Y, Chang V, Stoimenov PK, Schimel JP, Stucky GD, *et al.* (2013). Assessing interactions of hydrophilic nanoscale TiO2 with soil water. *J Nanopart Res* **15**:1–13.

Prosser JI, Nicol GW. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology* **20**:523–531.

Raes J, Bork P. (2008). Molecular eco-systems biology: towards an understanding of community function. *Nat Rev Micro* **6**:693–699.

Rico CM, Majumdar S, Duarte-Gardea M, Peralta-Videa JR, Gardea-Torresdey JL. (2011). Interaction of Nanoparticles with Edible Plants and Their Possible Implications in the Food Chain. *J Agric Food Chem* **59**:3485–3498.

Robichaud CO, Uyar AE, Darby MR, Zucker LG, Wiesner MR. (2009). Estimates of Upper Bounds and Trends in Nano-TiO2 Production As a Basis for Exposure Assessment. *Environ Sci Technol* **43**:4227–4233.

Rousk J, Ackermann K, Curling SF, Jones DL. (2012). Comparative Toxicity of Nanoparticulate CuO and ZnO to Soil Bacterial Communities. *Plos One* **7**:e34197.

Roux-Michollet D, Czarnes S, Adam B, Berry D, Commeaux C, Guillaumaud N, *et al.* (2008). Effects of steam disinfestation on community structure, abundance and activity of heterotrophic, denitrifying and nitrifying bacteria in an organic farming soil. *Soil Biology and Biochemistry* **40**:1836–1845.

Schimel JP, Bennett J, Fierer N. (2005). Microbial community composition and soil nitrogen cycling: is there really a connection? *Ecol Rev* 171–188.

Schimel JP, Schaeffer SM. (2012). Microbial control over carbon cycling in soil. *Front Microbiol* **3**. doi:10.3389/fmicb.2012.00348.

Schloter M, Dilly O, Munch JC. (2003). Indicators for evaluating soil quality. *Agric Ecosyst Environ* **98**:255–262.

Semple KT, Doick KJ, Jones KC, Burauel P, Craven A, Harms H. (2004). Peer Reviewed: Defining Bioavailability and Bioaccessibility of Contaminated Soil and Sediment is Complicated. *Environ Sci Technol* **38**:228A–231A.

Servin A, Elmer W, Mukherjee A, Torre-Roche RD la, Hamdi H, White JC, *et al.* (2015). A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. *J Nanoparticle Res* **17**:1–21.

Shoults-Wilson WA, Reinsch BC, Tsyusko OV, Bertsch PM, Lowry GV, Unrine JM. (2011). Role of Particle Size and Soil Type in Toxicity of Silver Nanoparticles to Earthworms. *Soil Sci Soc Am J* **75**:365.

Simon-Deckers A, Loo S, Mayne-L'hermite M, Herlin-Boime N, Menguy N, Reynaud C, *et al.* (2009). Size-, Composition- and Shape-Dependent Toxicological Impact of Metal Oxide Nanoparticles and Carbon Nanotubes toward Bacteria. *Environ Sci Technol* **43**:8423–8429.

Simonin M, Richaume A. (2015). Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review. *Environ Sci Pollut Res* 1–14.

Sohm B, Immel F, Bauda P, Pagnout C. (2015). Insight into the primary mode of action of TiO2 nanoparticles on Escherichia coli in the dark. *Proteomics* **15**:98–113.

Solovitch N, Labille J, Rose J, Chaurand P, Borschneck D, Wiesner MR, *et al.* (2010). Concurrent Aggregation and Deposition of TiO2 Nanoparticles in a Sandy Porous Media. *Environ Sci Technol* **44**:4897–4902.

Som C, Berges M, Chaudhry Q, Dusinska M, Fernandes TF, Olsen SI, *et al.* (2010). The importance of life cycle concepts for the development of safe nanoproducts. *Toxicology* **269**:160–169.

Sun P, Shijirbaatar A, Fang J, Owens G, Lin D, Zhang K. (2015). Distinguishable Transport Behavior of Zinc Oxide Nanoparticles in Silica Sand and Soil Columns. *Sci Total Environ* **505**:189–198.

Sun TY, Gottschalk F, Hungerbühler K, Nowack B. (2014). Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environ Pollut* **185**:69–76.

Thio BJR, Zhou D, Keller AA. (2011). Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles. *J Hazard Mater* **189**:556–563.

Tian Y, Gao B, Silvera-Batista C, Ziegler KJ. (2010). Transport of engineered nanoparticles in saturated porous media. *J Nanoparticle Res* **12**:2371–2380.

Tian Y, Gao B, Wang Y, Morales VL, Carpena RM, Huang Q, *et al.* (2012). Deposition and transport of functionalized carbon nanotubes in water-saturated sand columns. *J Hazard Mater* **213–214**:265–272.

Tourinho PS, van Gestel CAM, Lofts S, Svendsen C, Soares AMVM, Loureiro S. (2012). Metal-based nanoparticles in soil: Fate, behavior, and effects on soil invertebrates. *Environ Toxicol Chem* **31**:1679–1692.

Tourna M, Stieglmeier M, Spang A, Könneke M, Schintlmeister A, Urich T, *et al.* (2011). Nitrososphaera viennensis, an ammonia oxidizing archaeon from soil. *PNAS* **108**:8420–8425.

Tsuzuki T. (2009). Commercial scale production of inorganic nanoparticles. *Int J Nanotechnol* **6**:567–578.

Van Straalen NM. (2003). Peer Reviewed: Ecotoxicology Becomes Stress Ecology. *Environ Sci Technol* **37**:324A–330A.

Vitorge E. (2010). Développement et utilisation de nanotraceurs pour l'étude du transport de colloïdes en milieu poreux. Expérimentations et Modélisations. phdthesis, Université de Grenoble https://tel.archives-ouvertes.fr/tel-00536043/document (Accessed July 19, 2015).

Vittori Antisari L, Carbone S, Gatti A, Vianello G, Nannipieri P. (2013). Toxicity of metal oxide (CeO2, Fe3O4, SnO2) engineered nanoparticles on soil microbial biomass and their distribution in soil. *Soil Biol Biochem* **60**:87–94.

Wang Y, Gao B, Morales VL, Tian Y, Wu L, Gao J, *et al.* (2012). Transport of titanium dioxide nanoparticles in saturated porous media under various solution chemistry conditions. *J Nanoparticle Res* **14**:1–9.

Whiteside MD, Treseder KK, Atsatt PR. (2009). The brighter side of soils: Quantum dots track organic nitrogen through fungi and plants. *Ecology* **90**:100–108.

Whitley AR, Levard C, Oostveen E, Bertsch PM, Matocha CJ, Kammer F von der, *et al.* (2013). Behavior of Ag nanoparticles in soil: Effects of particle surface coating, aging and sewage sludge amendment. *Environ Pollut* **182**:141–149.

Yang Y, Wang Y, Westerhoff P, Hristovski K, Jin VL, Johnson M-VV, *et al.* (2014). Metal and nanoparticle occurrence in biosolid-amended soils. *Sci Total Environ* **485–486**:441–449.

Zhalnina K, de Quadros PD, Camargo FAO, Triplett EW. (2012). Drivers of archaeal ammonia-oxidizing communities in soil. *Front Microbiol* **3**. doi:10.3389/fmicb.2012.00210.

Zhang Y, Chen Y, Westerhoff P, Crittenden J. (2009). Impact of natural organic matter and divalent cations on the stability of aqueous nanoparticles. *Water Res* **43**:4249–4257.

Zhou D, Abdel-Fattah AI, Keller AA. (2012). Clay Particles Destabilize Engineered Nanoparticles in Aqueous Environments. *Environ Sci Technol* **46**:7520–7526.